Development and implementation of a regional platelet diagnostic laboratory in order to enhance the diagnosis and treatment of inherited platelet function disorders.

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A portfolio of research and development in a professional context

This thesis is submitted in partial fulfilment of the requirements for the award of the degree of Doctorate in Biomedical Sciences of the University of Portsmouth September 2012

ABSTRACT

This project sets out to create a reference laboratory service capable of detecting platelet function disorders using the latest techniques and based on the most current research. Platelet function disorders are difficult to diagnose due to differing phenotypic presentation and multiple causative agents. Platelet research has moved rapidly over the past decade and has included new reagents, analysers and techniques in the way platelet function disorders are diagnosed. A forward-looking diagnostic laboratory needs translate this ongoing research into routine laboratory practice, whilst ensuring that techniques used in the laboratory comply with the current guidelines and performed in a standardised and scientifically rigorous way.

Literature searches where used to develop a pre-analytical questionnaire and this has been adopted. It has proved to be an important tool for standardisation of the pre-analytical procedure which is now in use at other diagnostic centres. New light transmission aggregation equipment and agonists have been introduced, standardised and reference ranges generated, driven by evidence based practice. Reagent comparison studies have been undertaken to asses' cost-effectiveness of the assays in the laboratory. Platelet nucleotide reference ranges have been generated and are in use. The flow cytometric analysis of glycoproteins has been brought 'in-house', has been standardised, and is now being offered as a routine assay to specialist haematology clinical staff, improving the service the laboratory offers.

This work has enabled an extended range of assays available to the laboratory and now has capacity for specialist testing of inherited platelet disorders. This together with expert clinical staff creates the scientific and technical environment required for the establishment of a specialist regional referral centre. The reputation of the laboratory

has been additionally enhanced, through presentations and collaborations with manufacturers, other healthcare scientists and professional bodies.

All these improvements based on strong scientific research and rigorous application have enabled the patient to undergo a thorough investigation with the minimum of inconvenience and enabled the health care provider to utilise resources more effectively.

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Abbreviations

A23187	Calcimycin
ADAMTS13	a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
ADP	adenosine diphosphate
APTT	activated partial thromboplastin time
AP3	Adapter protein 3
ATP	adenosine triphosphate
BLOC	biogenesis of lysosome related organelle complex
BCSH	British Committee for Standards in Haematology
Ca2+	calcium ions
CI	confidence interval
COX	cyclo-oxygenase
COX1	cyclo-oxygenase isomer 1
CST	cytometer set and test
DAG	diacylglycerol
DIC	disseminated intravascular coagulopathy
EDGF	endothelial derived growth factor
EDTA	Ethylenediaminetetraacetic acid
FII	Factor II (also known as prothrombin)
FIIa	activated Factor II (also known as thrombin)
FITC	Fluorescein isothiocyanate
FLU	fluorescent light unit
FSC	Forward scatter
FV	Factor V

FVa	activated Factor V
FVII	Factor VII
FVIIa	activated Factor VII
FVIIa-TF	activated Factor VIIa tissue factor complex
FVIII	Factor VIII
FVIIIa	activated Factor VIII
FX	Factor X
FXII	Factor XII
FXIIa	activated Factor XII
GPIb	Glycoprotein Ib (also known as CD42b)
GPIIb	Glycoprotein IIb (also known as CD41)
GPIIIa	Glycoprotein IIIa (also known as CD61)
GPIIb/IIIa	Glycoprotein IIb/IIIa dimer
GPVI	Glycoprotein VI
HIT	heparin induced immune thrombocytopaenia
HMWK	high molecular weight kininogen
HPA	Human Platelet Antigen
HPS	Hermansky Pudlak syndrome
ISTH	International Society for Thrombosis and Haemostasis
IT	information technology
ITP	idiopathic thrombocytpaenic purpura
KPI	key performance indicator
LAMP3	lysosomal activating membrane protein 3
LTA	light transmission aggregometry
μM	micromolar
MFU	mean fluorescence unit

mg/mL	milligrams per millilitre
MYH9	myosin heavy chain gene 9
OTC	over the counter
PAP-4D	BioData 4 channel platelet aggregation profiler
PAP-8E	BioData 8 channel platelet aggregation profiler
PAR	Protease activated receptor
PCR	polymerase chain reaction
PCV	packed cell volume
PDGF	platelet derived growth factor
PE	Phycoerythrin
PF4	platelet factor 4
PFA-100	platelet function analyser 100
PG2	prostaglandin G2
PIP2	phosphatidylinositol bisphosphate
PK	pre-Kallikrein
PKC	protein kinase C
PLC	phospholipase C
PLCγ	phospholipase C γ isomer
PMP	platelet microbial proteins
PPACK	D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone
PPP	platelet poor plasma
PRP	platelet rich plasma
PSGL1	P-selectin glycoprotein ligand 1
PT	Prothrombin time
RCPath	Royal College of Pathologists
ROTEM	rotational elastometry

SD	standard deviation
SEM	standard error of the mean
SOP	standard operating procedure
SPD	storage pool disorder
SSC	scientific subcommittee (specialist standing committees of the ISTH)
SSC	side scatter (when referring to flow cytometry data)
SSRI	selective serotonin release inhibitor
TF	tissue factor
TRAP	thrombin receptor agonist peptide
TTP	thrombotic thrombocytpaenic purpura
TXA_2	thromboxane A_2
U46619	9,11-Dideoxy-9a,11a-methanoepoxy prostaglandin F_{2a} a stable thromboxane analogue
UKHCDO	UK Haemophilia Centre Directors Organisation
u-PA	urokinase plasminogen activator
VASP	vasodilator-stimulated phosphoprotein
VEGF	vascular endothelial derived growth factor
VWD	von Willebrand disease
VWF	von Willebrand Factor
WAS	Wiskott Aldrich syndrome
WASp	Wiskott Aldrich syndrome protein

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To Dr Gary Moore I say, ‘I think I may have done it!’

Dissemination

Detailed below is the academic output to which I have contributed during the period of study

Posters

D Gurney, GW Moore, S Rangarajan

Platelet Glycoproteins: Predictors or progression markers in cardiovascular disease?

International Society of Thrombosis & Haemostasis Geneva 2007

B Madan, C Rea, A Dunkerley, **D Gurney**, D Holloway, L Chappel

Successful management of pregnancies in three sisters with varying phenotypes of von Willebrand's disease.

International Society of Thrombosis & Haemostasis Boston 2009

B Madan, G W Moore, K Brown, P Earnshaw, **D A Gurney**

Successful management of major surgery with IVIg in a patient with a progressive acquired von Willebrand factor inhibitor on the background of hereditary Type 1 von Willebrand disease

International Society of Thrombosis & Haemostasis Boston 2009

Presentations

D Gurney Flow Cytometry in the Haemostasis Laboratory

Institute of Biomedical Science Biennial Congress 2003

D Gurney Case Presentation

Institute of Biomedical Science Biennial Congress 2005

D Gurney Case Presentation

Institute of Biomedical Science Biennial Congress 2009

D Gurney Platelet analysis: Send in the clones

Institute of Biomedical Science Biennial Congress 2011

Papers

David Gurney Ask and you will find. Academic content on the internet

The Biomedical Scientist Feb 2006

GW Moore, AV Kumat, **DA Gurney**, O O'Connor, S Rangarajan, R Carr, GF Savidge

Alteration in the laboratory profile of a lupus anticoagulant in a patient with non-Hodgkins lymphoma

Clin Lab Haem 2004, 26, 429-434

David Gurney Biomedical Science in the Palm of your Hand

The Biomedical Scientist June 2004

David Gurney Platelets: Food for Thought

The Biomedical Scientist March 2004

Spencer CG, **Gurney D**, Feldmen DC, Blann AD, Beevers DG, Lip GY

The platelet and haemorheological markers in 'high risk' hypertensives are improved by tighter blood pressure control and cardiovascular risk management: a sub study of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT)

Journal of Internal Medicine 2004; 255: (1)

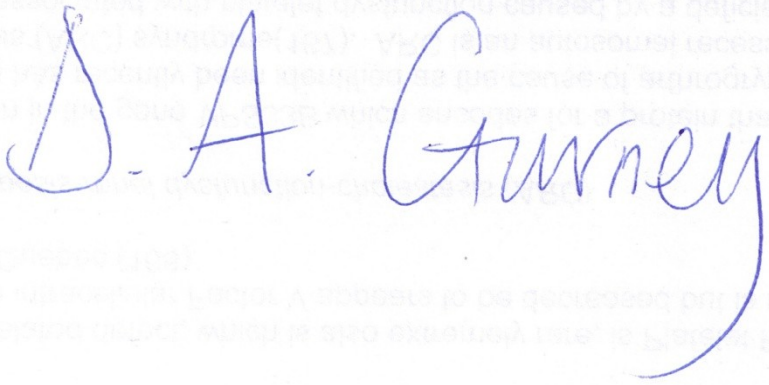
Book Chapter

Moore GW, Gurney DA Bleeding disorders and their laboratory investigation Haematology (Fundamentals of Biomedical Science) Moore, Blann and Knight OUP 2010

Declaration

Whist registered as a candidate for the above degree, I have not been registered for any other award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other award.

Signed



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Chapter 1: Introduction

Blood is a complex fluid composed of many constituent parts. The components of this system are leukocytes, erythrocytes, platelets and plasma. The areas on which this thesis will concentrate on are the platelets and plasma constituents (e.g. blood clotting proteins). Platelets have always played a crucial role in health and disease. Formed from the megakaryocyte in the bone marrow and circulating for ten days in the blood stream before removal by the spleen, they are sensitive to their local physical and chemical environment.

Platelets are principally involved in physiological haemostatic interactions, including those taking place between platelets and the blood vessels. Critical to these processes (which include adhesion aggregation and content release) are the platelet membrane and various organelles, including alpha granules, delta granules and lysozymes. The platelets also play a role in the localisation and control of many biochemical processes, such as prostaglandin production and the coagulation cascade. The latter process is particularly important for haemostasis and involves zymogens (enzyme precursors) and co-factors from the plasma. Various products, such as Factor V (FV) and von Willebrand factor (VWF) are also delivered from within the platelet, to the outer membrane and on to the local plasma environment. This creates a micro-environment allowing optimal zymogen interaction, as well as a level of protection from inhibitory processes where further clotting factors are activated driving the cascade forward.

These processes, as well as the dysfunction of platelets and laboratory investigation of platelet disorders will be the focus of this chapter.

1.1 Normal platelet function

1.1.1 Historical overview

Investigations into thrombus formation began in the mid 19th century (reviewed in (Gazzaniga & Ottini, 2001); (Mustard, J. F. Kinlough-Rathbone R.L., 2002) when both

Wharton-Jones (1852) and Zhan (1875) independently observed white thrombi in frogs. However as frog platelets have nuclei, they incorrectly stated that these were leukocyte fragments. Shultz (1865) observed platelets independently calling them 'clumps of irregular shape' and by 1873 Ranvier had described fibrin clots. In 1882, Hayem described the platelets ability to change shape and noted their 'spiney' nature. However it was the Italian Bizzozzero in 1882 who is credited with describing platelets as is understood today. In Bizzozzeros text he named them 'piasthne', which translated as 'petite plaques' or 'plaquettes' when the text was translated to French. The anglicising of the word plaquettes gives us *platelet.* In 1885 Lubnitsky described these new blood components as being part of the haemostatic plug. Platelet research then lulled until the late 1950s when Bournameaux described platelets ability to adhere to vascular subendothelium. Hovig advanced this research in 1963 to describe platelets ability to interact with collagen. Hellem and Owen postulated that erythrocytes released a substance that was able to aggregate platelets and in 1961 Gaarder proved that this was adenine diphosphate (ADP). The big leap in platelet research was facilitated by Gustav Born in 1962 and his invention of the platelet aggregometer (considered in more detail in section 1.3.3) (Born et al., 1963; Born, 1962).

This led to an acceleration in research in the mid 1960s; Born went on to show that platelet aggregation adopted Michelis-Menton like kinetics and second phase aggregation was described. Platelet ultra-structure was demonstrated by electron microscopy, Ardlie (1966) showed adrenaline to be a platelet aggregator and Kloeze (1966) described prostaglandin interaction. The prostaglandin interaction work led to aspirin being described as a potent cyclo-oxygenase inhibitor; work for which John Vane was awarded the Nobel Prize in 1970.

1.1.2 Platelets in primary haemostasis

The haemostatic process is nominally broken down into two distinct phases, primary haemostasis and secondary haemostasis.

Primary haemostasis are the actions leading to the initial plugging of the breached vessel. This involves platelets, the vessel wall and von Willebrand factor (VWF). Normal flow (or shear rate) is maintained by the vessel releasing nitric oxide (NO) and prostaglandin I_2 (PGI_2). When there is a break in the vessel, sub-endothelial collagen is exposed and the concentration of PGI_2 and NO decreases and the vessel constricts in response. This slows the blood flow which minimises blood loss and increases the time in which thrombotic components are in contact with the vessel wall.

Blood vessels contain collagen types I – IV and VI however it is types I and III that are involved in adhesion. In vessels such as veins and the large arteries, with a low flow rate, and therefore a low shear stress, the exposed sub-endothelial collagen binds directly to the glycoprotein Ia/IIa (GPIa/IIa) located on the platelet membrane to tether it then Glycoprotein VI (GPVI) (Varga-Szabo, Pleines, & Nieswandt, 2008) activates receptors on the platelets membrane surface. In vessels with a higher shear rate, such as the arterioles the von Willebrand factor (VWF) is needed to anchor the platelets. VWF interacts with the type I collagen, which causes a conformation change that causes adhesion to the platelet GPIa, then as in low shear environments GPVI activates the platelet.

As the platelet activates and shape change is initiated glycoprotein Ib (GPIb) binds to VWF and anchors the platelets to the endothelial surface. When there is a break in the integrity of the endothelium, VWF is released from storage in the endothelial Weibel-Palade bodies into the circulation. This activates the GPIb component of the GPIb-V-IX complex, and VWF is immobilised on exposed collagen fibrils, facilitating the activation of receptors GPIa/IIa and GPVI on the platelet surface(Gardiner et al., 2010).

GPIb activates phospholipase C (PLC) which in turn catalyses phosphatidylinositol bisphosphate (PIP2). This produces diacylglycerol (DAG) and phospholipase A_2 (PLA_2). DAG is the substrate for protein kinase C (PKC) and the product of this reaction interacts with the tail portion of GPIIbIIIa and the receptor change enhances the binding of fibrinogen to GPIIbIIIa. Cross linking of GPIIbIIIa on other platelets by fibrinogen causes irreversible aggregation to proceed. PLA_2 catalyses membrane phospholipid to arachidonic acid, which itself is catalysed by cyclo-oxygenase to become prostaglandin G_2 (PG_2). PG_2 is catalysed by thromboxane synthase to thromboxane A_2 (TXA_2). This is released and reacts with the thromboxane receptor amplifying the cascade mechanism.

The collagen-activated GPVI catalyses a different isomer of PLC (PLCγ2) to achieve the amplification of the release cascade. As these reactions take place calcium ions are mobilised through the platelet via the dense tubular system. These calcium ions contribute to the release of thromboxane and to the fusion of granules with the external membrane.

Alpha granules, following fusion with the main platelet membrane (Gurney, Lip, & Blann, 2002), expose P-selectin on the outer surface of the platelet. The P-selectin was previously contained in an inactive form on the internal surface of the granules. P-selectin facilitates the adhesion of platelets to leukocytes and vessel surfaces. The granule contents are also released into the micro-environment. These contents include fibrinogen and GPIIbIIIa, (which enhances further GPIIbIIIa coverage and consequently additional binding) and VWF (which will amplify tethering and platelet/platelet binding) and a number of growth factors (platelet derived growth factor (PDGF), endothelium derived growth factor (EDGF), and vascular endothelial growth factor (VEGF) required for repair to the damaged vasculature. During this period, dense granules are also released. These contain secondary amplification products such as

calcium ions, serotonin (5HT), ADP and adenosine triphosphate (ATP). Calcium ions increase the external concentration increase the flux across the platelet membrane to drive thromboxane generation. Serotonin constricts the vessel wall to decrease blood loss from the damaged vessel and ADP activates the $P2Y_1$ and $P2Y_{12}$ receptors on the platelet. These receptors are Gq- and Gi- coupled receptor respectively, which feed into the PKC pathway detailed above. They also release more P-selectin and GPIb onto the platelet membrane surface.

Due to forward flow the platelet continues to roll along the surface causing more collagen adhesion and VWF binding. The platelet also changes shape, rearranging its actin-myosin skeleton from discoid to spherical and finally extending filopodia, to facilitate attachment to the damaged endothelium. Eventually the platelet is tightly bound to the surface of the break in the vessel. These events do not continue in isolation; the platelet has released molecules from cellular granules into the localised micro-environment.

This serves to recruit additional platelets to plug the break in the vessel and stem the loss of further blood. These additional platelets are bound to the original activating platelet, as activation of GPIa increases the affinity of glycoprotein IIb/IIIa (GPIIb/IIIa) for fibrinogen, allowing the rapid cross linking of platelets to form the characteristic fibrin mesh. This mesh eventually involves other blood cells captured within the fibrous structure to give the recognisable ‘blood clot’ (Figure 1.1).

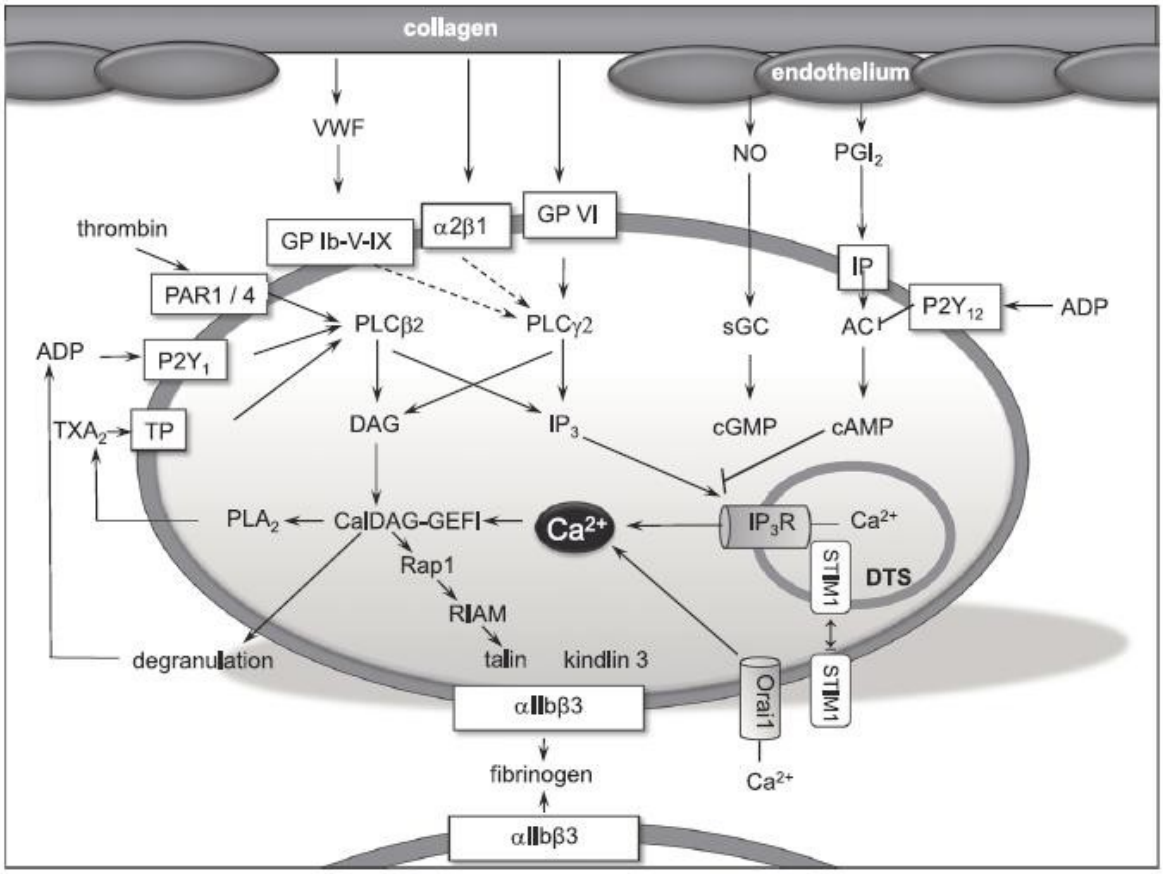

Figure 1.1 Overview of platelet biochemistry Used with permission. See reference for legend (Broos et al., 2012)

1.1.3 Platelets and the coagulation cascade (Secondary haemostasis)

Simultaneously to the platelets being activated, the clotting mechanism is initiated. This process of secondary haemostasis involves the action of a series of enzymes, aided by co-factors such as FVIII, in the fluid component of the blood - converting it from a liquid form into a solid localised clot. This is partially achieved by the release of tissue factor (TF) from the platelet membrane and further tissue factor exposure from the adventitia (outer) layer of the blood vessel. The released tissue factor binds with Factor VII (FVII) and to its activated form FVIIa. The FVII remains inactive until activated by the FVIIa-TF complex. This complex can reside on the platelet surface, where it will bind Factor X (FX) to form the extrinsic tenase complex.

Simultaneous to the extrinsic tenase complex formation, there is intrinsic tenase

complex production, it is comprised of activated Factor IX (FIXa), activated Factor VIII (FVIIIa), Factor X (FX) and calcium ion (Ca^{2+}). The FVIII has been made available by the platelet alpha granules and plasma, and the Ca^{2+} provided by the delta (dense) storage granules. However FX is activated, it - together with activated Factor V (FVa) - complexes with Factor II (prothrombin) converting the inactive zymogen into the active enzyme thrombin (FIIa). This converts soluble fibrinogen to insoluble fibrin causing the haemostatic mesh to form. The thrombin also activates the protease activated receptors (PARs) on the platelet surface. This is the final stage in creating the 'plug'. Both 'tenase' and 'prothrombinase' complexes need a phospholipid surface and this is provided by the platelet membrane, facilitated by the exposure of phosphatidylserine within discrete vesicles (Lentz, 2003). The platelet provides the delivery system, the scaffold system and much of the bulk for the coagulation cascade.

These processes have been studied in great detail, initially to understand the cause of bleeding disorders but also to seek methods for reducing the incidence of cardiovascular disease and prevent inappropriate thrombus formation.

Vascular damage/disruption also initiates the process of contact activation. High molecular weight kininogen (HMWK) and pre-Kallikrein (PK) are present in plasma and activated by activated Factor XII (FXIIa). This activation is in response to an external activator such as change in the vessel surface or change of pH. The activated HMWK and Kallikrein bind to platelets via the GPIb complex. Concurrently platelet dense granules release inorganic polyphosphate (Ruiz, Lea, Oldfield, & Docampo, 2004) which has been shown to accelerate the activation of FXI by thrombin and FXIa (Choi, Smith, & Morrissey, 2011) This shows that platelets are involved at the very fulcrum of the coagulation mechanism. Understanding the function of platelets can lead the understanding of other coagulation disorders further down the cascade.

1.1.4 Other roles for platelets

By virtue of the platelets' involvement with delivery of prostaglandins and interactions with the vasculature; researchers questioned whether the platelet was involved in other physiological processes. It has been found that platelets do play an integral role in the immune response, including interactions with leukocytes. It has been shown that the platelet contains over three hundred unique proteins (Coppinger et al., 2004), with most being contained within the alpha granules.

When the platelet is activated and the alpha granules fuse with the platelet membrane and these bioactive molecules are released into the local microenvironment. There are molecules involved in vascular remodelling, such as histidine rich glycoprotein and α-2-antiplasmin. There are those that are involved in angiogenesis such as C1-inhibitor and α-1-antitrypsin. Vascular endothelial growth factor (VEGF) and Platelet derived growth factor (PDGF) are involved in cell proliferation.

The platelet specific receptor P-selectin is exposed from the inner membrane of the alpha granule to the outer membrane of the platelet as the granule fuses with the outer envelope of the platelet. One of the many ligands for this is the P-selectin glycoprotein ligand 1 (PSGL1) which is expressed exclusively on leukocyte membranes. Once tethered to platelets, these neutrophils bind via tightly via GPIIIa, and cause production of thromboxane and prostaglandins (Weyrich, Lindemann, & Zimmerman, 2003).

In addition, they are also involved in the initial mechanical phase when encountering foreign bacteria and viruses. They have been shown to engulf them, producing viricidal and fungicidal chemicals called platelet microbial proteins (PMPs) predominantly containing two groups of thrombocidins (induced by thrombin) and kinocindins (a dual chemokine and microbiological entity), and reactive oxygen species (Yeaman, 2010).

The alpha granules also contain the serine protease plasmin and its zymogen precursor plasminogen, antithrombin and C1-inhibitor. Plasmin breaks down fibrin, antithrombin

binds to thrombin, and C1-inhibitor reduces the efficacy of the contact activation system. The platelet does not actively contribute to the fibrinolytic process; however it does release products that arrest the procedure and keep balance (Blair & Flaumenhaft, 2009).

Dense granules contain calcium ions, which apart from being essential to the coagulation cascade are also required for would healing and fibrin formation and serotonin a potent vasoconstrictor which will modulate blood flow through the damaged vessel and increase capillary permeability. This increase in permeability allows cellular components to pass through to the lymph. Platelets also have receptors that induce B-cells to produce antibodies and enhance T-cell responses (Elzey et al., 2003)

1.2 Platelet dysfunction in bleeding disorders

1.2.1 Platelet quantitative disorders

Platelets contribute to a wide variety of processes as shown previously. Therefore a reduction in platelets in diseases such as idiopathic thrombocytopaenic purpura (ITP) and thrombotic thrombocytopaenic purpura (TTP) gives bleeding symptoms as there are insufficient platelets to perform the functions required. TTP can be genetic, where there is an absence of a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13(ADAMTS13) and acquired, where an event has instigated a blocking of ADAMTS13 function. These events can range from pregnancy, cancer, systemic lupus erythematosus and organ transplantation.

There are a numbers of disorders that are functional defects, such as Bernard Soulier syndrome and Myosin Heavy chain gene 9 (MYH9) disorders, which presents with a low platelet count, but characteristically large platelets (macrothrombocytopaenia). As described above platelets use many receptors and release many bioactive components to

provide optimal function. Disorders can include reduction or absence in any of these.

MYH9 disorders (Althaus & Greinacher, 2009), such as May-Hegglin, Fechtner and Sebastian syndromes are multi-factorial disorders including symptoms such as nephritis, cataracts, deafness, leukocyte inclusions and macrothrombocytopaenia. The defect is within the actin myosin skeleton of the platelets outer envelope. The platelets are released from the megakaryocyte prematurely and have poor structural integrity.

1.2.2 Acquired platelet function disorders

There are various disorders that influence function as well as the quantity of platelets. These include genetic and acquired causes.

The largest acquired cause is drug-related, such as thrombocytopaenia caused by heparin. Heparin induced thrombocytopaenia (HIT) is an difficult disorder to diagnose and manage (Cuker et al., 2010; T. Warkentin, 2006). Abciximab (Aster, 2005) also causes thrombocytopaenia, this is characterised (compared to HIT) by its rapid onset and markedly low platelet count. Some systemic disorders affect platelet function, such as renal failure (Remuzzi et al., 1978), liver disease (Witters et al., 2008) and sepsis (Yaguchi et al., 2004).

Disseminated intravascular coagulopathy (DIC) -via fibrin/fibrinogen degradation products can down regulate platelet function (Yaguchi et al., 2004), this is due to both activation and consumption. Abnormal platelet function, as well as thrombocytopaenia, is sometimes seen associated with the chronic myeloproliferative disorders, acute myeloid leukaemia and myelodysplastic syndromes. Significant numbers of patients with myeloma and Waldenströms macroglobulinaemia have impaired platelet function due to the interference of function by paraproteins (Zangari et al., 2007). This is thought to be mediated by interference with fibrinogen polymerisation (Cohen et al., 1970)

Drugs are the most common cause of platelet dysfunction. Aspirin irreversibly inactivates the platelet cyclo-oxygenase pathway, thereby blocking aggregation and it

can therefore be used as an effective anticoagulant drug. Many other drugs are known to affect platelet function to varying degrees, as shown in Table 1.1:

Drug Type	Example	Action	Reference
NSAID	Aspirin, Ibuprofen	Inhibition of COX	(Gurbel et al., 2007)
Beta Lactam Antibiotics	Penicillin	Irreversible binding to the membrane, disruption of calcium flux.	(Cazenave, et al., 1973)
Cardiovascular drugs	Thienopyridines e.g. clopidogrel and prasgrul, RheoPro and	Competitive binding to membrane receptors	(Ben-Dor, Kleiman, & Lev, 2009)
Psychotropics	SSRIs,	Competitive binding to adreno-receptors and reduction in serotonin pool	(Dietrich-Muszalska et al., 2010)
Anaesthetics	Halothane	Reduction in Ca^{2+} flux	(Kohro & Yamakage, 1996)
Chemotherapeutic agents	Thalidomide for myeloma	Thrombotoxicity	(Kwaan & Vicuna, 2007)
Anticoagulants	Heparin	Binding to FC-receptor via PF4, leading to sequestration and thrombocytopaenia. (HIT)	(T. E. Warkentin & Greinacher, 2004)

Table 1.1: Drugs that interfere with platelet aggregation studies

Not every drug within each category will affect platelet function and their effects tend to be concentration-dependent.

Some foods and food additives can also affect platelet function if ingested in sufficient amounts. Examples include fish oils (Din et al., 2008), green tea (Son et al., 2004), garlic (Iciek, Kwiecień, & Włodek, 2009), cumin (Srivastava, 1989), turmeric (Cordier & Steenkamp, 2012), chocolate (Khawaja, Gaziano, & Djoussé, 2011), excessive use of vitamin supplements (Kobzar et al., 2009; Violi, Pignatelli, & Basili, 2010), and the black tree fungus (Kim et al., 2011) used in Chinese cuisine. In sufficient amounts, these foodstuffs can generate abnormal results in platelet function testing that can

complicate diagnostic interpretation. However, abnormal results due to foodstuffs alone are unlikely to translate into a genuine acquired bleeding disorder.

1.2.3 Genetic Platelet disorders

As presented above there are a number of ways the haemostatic mechanism involving platelets can break down. Genetic disorders of platelet function are characterized by the area of function affected, as follows.

1.2.3.1 Major deficiencies of surface adhesion receptors

The two most common receptor disorders are Glanzmann's thrombasthenia and Bernard–Soulier syndrome, which are amongst the most clinically severe of the hereditary platelet function defects.

First described by Swiss paediatrician Eduard Glanzmann (Glanzmann, 1918)

Glanzmann's thrombasthenia is characterized by a deficiency or functional defect of the GPIIb/IIIa complex first elucidated independently by Nurden and Phillips (Nurden & Caen, 1974; Phillips et al., 1975). This complex is the product of proteins from two genes ITGA2B (GPIIb) and ITGB3 (GPIIIa), these genes are located on chromosome 17. There have been 82 mutations characterised in ITGA2B and 51 in ITGB3 (Franchini, Favaloro, & Lippi, 2010), the discrepancy due to the ITGA2B gene being larger (65kbp compared to 17kbp). These are registered on the Glanzmanns Thrombasthenia database (http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu).

Flow cytometric analysis of the glycoproteins was first described almost 30 years ago (Jennings et al, 1986)

It is classified into three subtypes (George, Caen, & Nurden, 1990):

• Type I has 0–5% of normal levels of GPIIb/IIIa receptor numbers on the platelet surface

• Type II has 6–20% GPIIb/IIIa of normal levels of GPIIb/IIIa receptor numbers on the platelet surface

• 'Variant' has 50–100% GPIIb/IIIa receptor numbers on the platelet surface but with reduced fibrinogen binding.

The most common clinical features are typical of a primary haemostatic disorder, such as epistaxis, purpura, petechiae, excessive bruising, gum bleeding and menorrhagia. Platelet counts are normal. Bleeding after trauma/surgery can be severe and pregnancy/delivery markedly increase the risk of bleeding (Siddiq, Clark, & Mumford, 2011).

Two French haematologists Jean Bernard and Jean-Pierre Soulier described this disease in 1948 (Bernard & Soulier, 1948) Bernard–Soulier syndrome results from the absence or decreased expression of the GPIb–V–IX complex on the platelet surface. Patients present with mild to moderate thrombocytopaenia and enlarged or giant platelets. Bleeding symptoms are also typical of a primary haemostatic disorder, but are more severe than expected for the degree of thrombocytopaenia due to the concomitant functional defect. Bernard-Soulier syndrome is a result of a mutation in one of three genes. Gene GP1BA encodes the proteins for the alpha sub-unit of GPIb, GP1BB encodes for the beta sub-unit and GP9 for the GPIX.

1.2.3.2 Other receptor defects

Defects in the coupled ADP receptor P2Y12 is an autosomal recessive disorder first described by Cattaneo in 1992 (Cattaneo et al., 1992). These patients have defective ADP binding to the receptor due to defective receptor synthesis(Marco Cattaneo, 2005) Laboratory presentation includes reduced aggregation responses to ADP even at high doses such as 100μM, reversible aggregation to weak agonists and failure of nucleotide secretion (Podda, Femia, Pugliano, & Cattaneo, 2012)

There are two thromboxane receptors in human tissue, differing only in carboxy-terminus. This is caused by a differential splicing of the TxA2R products and these are called TxA2Rα and TxA2Rβ respectively. Only TxA2Rα appears on platelet surfaces. These receptors play a critical role in up-regulation of the platelet aggregation cascade, responding to the liberation of arachidonate from the platelet membrane and its subsequent conversion to thromboxane A_2. Defects have been demonstrated in this receptor (Mumford et al., 2010) giving a reduced response to both arachidonate and U46619.

Of the collagen receptor defects only GPVI has been described. This is normally caused by either autoimmune antibodies causing internalisation or shedding of the receptor (Bender et al., 2010), or mutations in the GP6 gene (Dumont et al., 2009) of unknown inheritance. Distinguishing GPVI defects in the laboratory is relatively easy using the agonist selective for the GPVI receptor, Convulxin. This is derived from the venom of the tropical rattlesnake *crotalus durissus terrificus* (Polgár et al., 1997). An Arg60 to Leu substitution has been found in the thromboxane A2 receptor (Hirata et al., 1994). With new agonists such as the thromboxane mimetic U46619 this is a relatively easy laboratory phenotypic diagnosis. Defects in the epinephrine receptor have been described (Kambayashi et al., 1996; Rao et al., 1988).

1.2.3.3 Platelet granule disorders

Platelet granule disorders are defined as a group of disorders where by platelet function is defective if granule numbers are reduced, granule contents are deficient, or if release mechanisms fail (Nurden, Freson, & Seligsohn, 2012). Most of these disorders affect dense (or δ) granules or α-granules, but rarely affect both. Since the dense and α-granules are storage sites, their defects are also referred to as storage pool disease.

Dense body disorders

Disorders of dense bodies are often part of a more complex congenital disorder, although isolated dense body defects are known. The bleeding phenotype associated with dense body disorders is usually of mild–moderate severity, with significant bleeding associated with trauma/surgery. Platelet counts and size are normal. There are three main disorders:

Hermansky–Pudlak (Walker et al., 2007) syndrome is a diverse autosomal-recessive disorder affecting a number of organelles, in particular, melanosomes and platelet dense bodies. There are nine identified variations of HPS, mutations in the genes coding for Hermansky Pudlak protein 1 and 4 being the most common and most severe (Masliah-Planchon et al., 2012). Hermansky Pudlak proteins cluster to form 'biogenesis of lysosome related organelle complex' (BLOC). These complexes HPS proteins 7, 8 and 9 cluster to form BLOC1. Proteins 3, 5 and 6 form BLOC2 and 1 and 4 for BLOC3. HPS protein 2 is part of the adapter protein 3 (AP3) complex, this complex is responsible for mediating recognition and protein sorting. The defect has been shown to cause lysosomal membrane proteins such as LAMP3 to be routed to the platelet outer envelope rather than into the dense body. A mutation in HPS2 gene leads to immunodeficiency along with the other associated pathologies such as albinism and dense body deficiency. These proteins are ubiquitous amongst cells but seem only to cause a defective phenotype in melanocytes (melanosomes) and platelets (dense bodies).

Chediak–Higashi (Buchanan & Handin, 1976) syndrome is an autosomal-recessive disorder also associated with albinism and dense body deficiency. It is caused by a mutation in LYST (CHS1) gene. This gene codes for a cytosolic protein thought to be involved in granule size and movement (Kaplan, De Domenico, & Ward, 2008).

Phenotypically the immune system is affected and large inclusion bodies are seen in white cell precursors in the bone marrow.

Primary dense body deficiency is a clinically heterogeneous disorder with an uncertain genetic basis that is not associated with other abnormalities. Within this group are the rare Rab27 disorders such as Griscelli syndrome. The Rab27a gene encodes for a protein involved in melanocyte and cytotoxic T-cell production. The mouse model has been found to have a reduced number of granules per platelet and an increased bleeding time, but no symptomatic bleeding diathesis. In human cases the T-cell mutation engenders a life threatening immunodeficiency. Recently a further subset has been described (Jedlitschky et al., 2010) with a mutation in the ABCC4 genes, the corresponding protein thought to mediate nucleotide trafficking into the granules.

Alpha-granule disorders

Defects of α-granules (Harrison & Martin Cramer, 1993) are extremely rare and so it is difficult to generalize about clinical features.

There are three main disorders:

Grey platelet syndrome is an autosomal recessive disorder linked with a complete absence of α-granules and thus the levels of their contents are reduced or absent. There are a number of genetic causes such as a mutation in the x-linked GATA1 gene (Paola, Johnson et al 2011; Tubman et al., 2007) and NBEAL2 (Albers et al., 2011). The resulting defective protein is unable to import proteins into the granule. This results in the characteristic appearance of platelets on Romanowksy stained blood films as agranular, misshapen grey ‘ghost’ platelets. The platelet count is often reduced and platelets can be slightly larger than normal (Bain & Bhavnani, 2011). Mass spectrometric analysis has shown that grey platelet syndrome platelets contain almost no PF4, βTG, Multimerin and fibronectin, but only a reduced amount of albumin and

fibrinogen. Some proteins that should be in the α-granule appear on the granule surface giving the possibility of mis-targeted protein production (Maynard et al., 2010). Paris-Trousseau syndrome (Favier et al., 2003) is characterized by thrombocytopaenia together with other congenital abnormalities, such as immune deficiency, neurological dysfunction and variable albinism. There are giant α-granules in a percentage of the circulating platelet population that cannot release their contents, and a sub-population of abnormally matured micro-megakaryocytes that lyse upon maturation. It is caused by a 11q chromosome deletion. Chromosome 11q contains the genes for ETS1 and FLI1, both of which have been demonstrated in megakaryocyte maturation. FLI1 especially activates genes encoding for GPIb, and this has been shown to relate to the pathology of the disease.

Quebec platelet disorder (Hayward & Rivard, 2011) previously known as Quebec factor V is characterized by increased levels of platelet urinary plasminogen activator (u-PA). This can be up to a one hundred fold increase. This increase in uPA, which activates platelet plasminogen, proteolysis platelet factor V along with other α-granule proteins and subsequently degrades α-granules content. It is an autosomal dominant disorder caused by the tandem deletion of PLAU on chromosome 10 (Blavignac et al., 2011). PLAU encodes for uPA and its regulatory proteins. Its laboratory profile is difficult to elucidate as plasma uPA is normal compared to platelet uPA, patients have a reduction in platelet count, compared to their unaffected kindred of about 50%. The platelet aggregation can show first phase only with epinephrine. The phenotypic bleeding can be attributed to reduced platelet proteins coupled with a reduced platelet count, but consensus is that it is due to increased fibrinolysis.

1.2.3.4 Disorders of platelet biochemistry

Abnormalities in platelet biochemistry inevitably impair platelet function. Wiskott–Aldrich syndrome (WAS) (Notarangelo, Miao, & Ochs, 2008) is a rare X-linked disorder resulting from defects in the *WAS* gene that encodes for the WAS protein (WASp). WASp is a regulator of actin polymerisation that has five defined domains. It has been shown to take part in biochemical signalling, synapse formation, cell locomotion and cytoskeleton maintenance. Children born with WAS present with bruising, small platelets and purpura resulting from thrombocytopaenia and abnormal platelet function. WAS is a complex disorder with features including eczema and immune deficiencies, which can be severe (Ochs & Thrasher, 2006).

Deficiencies of platelet enzymes such as cyclo-oxygenase have also been described (A. Nurden, Fiore, Pillois, & Nurden, 2009).

1.2.3.5 Disorders of phospholipid exposure

Scott syndrome is an extremely rare bleeding disorder characterized by the reduced exposure of negatively charged phospholipids to facilitate tenase and prothrombinase formation. Consequently the coagulation cascade cannot proceed to completion (Satta et al., 1997). Stormorken syndrome is also rare and is almost the reverse of Scott syndrome in that non-activated platelets express full procoagulant activity but a reduced response to collagen. Paradoxically this does not lead to a prothrombotic stage, but a mild bleeding disorder. This appears to stem from the inability to bind to collagen upon activation. Also there is increased clearance of the affected platelets, leading to mild thrombocytopaenia (Solum, 1999).

1.3 Overview of laboratory tests for platelet function disorders

1.3.1 Pre analytical factors

Before proceeding to laboratory investigation it is essential that the physician should examine the patient and take a detailed bleeding history (including type, location and frequency) and family history. As with any test, pre-analytical variables need consideration, such as the quality of the venepuncture. Ultimately such variables can distort the results and hinder interpretation. A clean draw using a wide bore needle (minimum 21G), giving minimal stasis and therefore minimal activation, is optimum. The blood should be collected into plastic or siliconised glass containers to minimise the activation by the charged surfaces (Vasin et al., 2003), a discard tube used to minimise inclusion of cellular activators such as tissue factor (Harrison et al., 2011),and consideration should be given to the anticoagulant. Sodium citrate has been demonstrated to enhance the effects of ADP and reduce the efficacy of GPIIb/IIIa (Kaiser et al., 2011)Other alternatives such as heparin, hirudin (Wallen et al., 1997) and D-Phenylalanyl-L-Prolyl-L-Arginine Chloromethyl Ketone (PPACK) are available.

Steps must therefore be taken to minimise the effects of confounding factors, including those indicated above (see Table 1.1). This extends to other lifestyle factors such as: exercise before analysis (Vucinic et al., 2010), smoking (Pamukcu et al., 2011) and family history (Borhany et al., 2010). Medical procedures performed in the recent past also need careful consideration, as products such as X-Ray contrast media (Heptinstall et al., 1998) will affect platelet function.

1.3.2 Point of care devices

The physiology of the blood and the vessel wall and the relationship between them was studied by Virchow in 1845 (Dickson, 2009). He proposed the association between the blood, the vessel wall, and the flow of the blood over the endothelium.

An overall measure of the mechanisms of the triad of *in vivo* vascular integrity is the bleeding time. This previously popular screening test for primary haemostatic disorders entails - when using the Ivy Method - the applying of a standardised pressure with a sphygmomanometer to the patient's upper arm and making a standardised cut in the inside of the forearm. Blood is removed from the wound site with a filter paper, so that a clot does not form, and the time for the bleeding to stop is measured. However the method has disadvantages, such as operator variability, dependence on fibrinogen levels, incision size, skin quality and skin temperature giving the assay poor reproducibility (Lehman et al., 2001; Rodgers & Levin, 1990). In diseases such as hereditary telangiectasia, the bleeding time is of diagnostic significance as the disease involves the breakdown in vascular integrity.

In relation to its haemostasis role, some centres have proposed the discontinuation of the bleeding time completely, in favour of a more reproducible mechanical method using whole blood. The Platelet Function Analyser (PFA-100, Dade Behring, Illinois, USA) (Kundu et al., 1995) analyses the platelets under stress of movement, passing a whole blood sample under negative pressure up a tube to an aperture, which is coated with either collagen and epinephrine, or collagen and ADP. The time taken for the platelets to plug this aperture is the 'closure time' and is measured in seconds. This assay cannot function with low platelet or packed cell volume (PCV) values. The assay, performed by using cartridges that are impregnated with either collagen and epinephrine or collagen and ADP, puts the blood under a pneumatic pressure. However, although this method satisfies two of Virchows' criteria, it provides no indication of vascular integrity (Li et al, 1998).

The VerifyNow (Accumetrics, California, USA) (Smith et al., 1999) is a system based on polymeric beads that are coated with either fibrinogen for testing GPIIb/IIIa inhibitors or arachidonate for the aspirin responder assay. It was designed to function as

a point-of-care (POCT) analyser and therefore requires minimal training. The analyser provides very specific information and is only approved for aspirin resistance screening and anti-glycoprotein therapy monitoring.

Cone and plate(let) analysis (Varon et al., 1997) also involves the use of applied pressure. The blood sample is placed in the analyser in which it is subjected to the equivalent of arterial sheer flow. Under the pressure applied, the blood sample passes across a plastic matrix, and after two minutes the matrix is stained and analysed by an image scanner. The size distribution, surface coverage, and average size is calculated and represented in numerical form. This has been commercialised and is the basis of the Diamed Impact analyser (Diamed, Cressier sur Morat, Switzerland).

At the 2004 International Society of Thrombosis and Haemostasis Scientific Standardisation Committee meeting, Diamed (Diamed, Cressier sur Morat, Switzerland) released the Impact R analyser (Shenkman et al, 2008) which gives researchers the ability to change the pressure characteristics, so that the effect of different shear rates are investigated.

Both the VerifyNow and the Impact analysers are reliant upon the respective agonists and the quality of the samples. Both the analysers suffer from the inherent limitation of other stand-alone platelet function analysers, such as the PFA-100, in that they are not able to measure vascular involvement. The clot signature analyser (Xylum, USA) (Li et al., 1998) based on work by Gőrőg (Görög & Ahmed, 1984) attempts to overcome this by using a method whereby the whole blood sample is passed through a vessel in the analyser that is subsequently punctured. The flow of the blood through the vessel and out of the puncture site is measured together with the thrombi build-up. This produces a characteristic plot or ‘signature’. This has the advantage that the measurement is taken over a period of time, not just a ‘snapshot’, as in the tests mentioned earlier

1.3.3 Light Transmission Aggregometry (LTA)

Based on the method developed by Born in 1962 (Born, 1962), platelet aggregation employs a light path through platelet rich plasma (PRP) and records the increase in transmission as the platelets are removed from suspension, as agglutination and aggregation take place. Agonists such as ADP, epinephrine and collagen are added to the PRP, and then the response of the platelets is monitored graphically.

This technique has its advantages and limitations. Although this process includes no vascular or other cellular involvement, it has value in demonstrating platelet disorders and diagnosing von Willebrand disease (VWD). Limitations include a lack of sensitivity to platelet aggregates, and the process is affected by stir speed, temperature, plasma lipids, pH changes, and processing time. Whole blood aggregation (Matsuno, Furukawa, & Terada, 1984) tests the platelet in a more physiological cellular environment, so that leukocyte and erythrocyte interactions are monitored; however it still does not show the effect of endothelial dysfunction or micro-aggregate formation and few centres use this method. Laser aggregometry (Toshima et al., 2008) was developed (PA-200, Kowa, Japan; 230ELA-2, Biola, Russia) that can detect aggregates much smaller than those detected by conventional light transmission aggregometers; however only one specialist research centre in the UK (Queens Medical Centre, Nottingham) has access to this equipment.

Routine agonists used are ADP, collagen, arachidonate, epinephrine and ristocetin, two further agonists TRAP and U46619 have recently been advocated for inclusion in the testing repertoire (Harrison et al., 2011). A standard trace detailing the phases seen in LTA is shown in figure 1.2

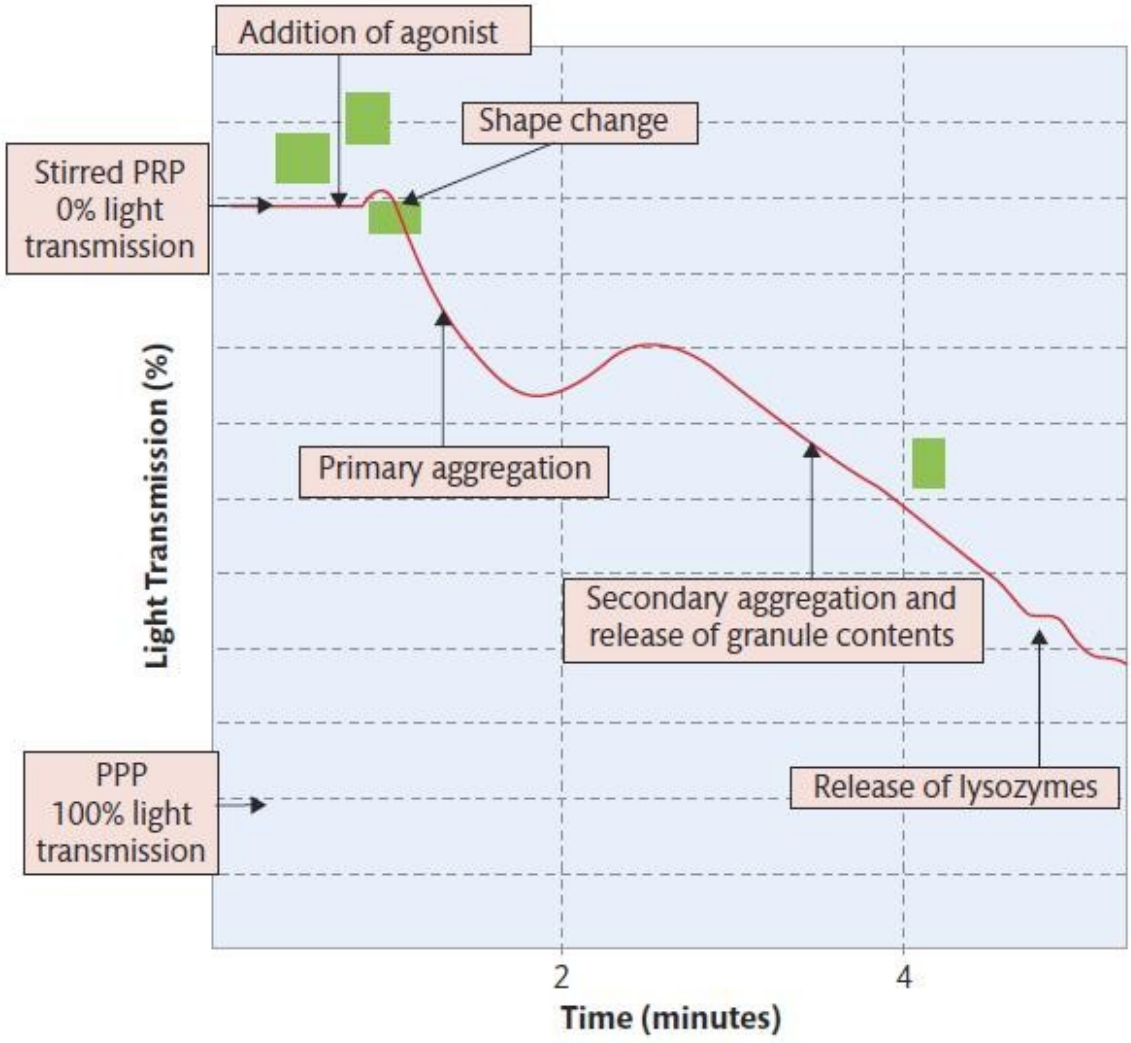

Fig 1.2: Stages of platelet agglutination and aggregation.

Aggregation trace showing the distinct phases of platelet aggregation and how these phases relate to platelet physiology

Used with permission from (Moore & Gurney, 2010)

1.3.3.1 Current agonists

Adenosine diphosphate:

ADP is a weak agonist at low concentrations. It therefore stimulates platelets to release other substances to continue to full aggregation. At higher concentrations these secondary releases are not required and aggregation proceeds fully. Using ADP as an agonist evaluates the platelets $P2Y_1$ and $P2Y_{12}$ receptors. These receptors induce the

release of further ADP from dense granules in the platelet and the formation of thromboxane A_2.

Arachidonate:

Arachidonate is released from the platelet membrane by phospholipase A_2. It is then converted to ProstaglandinG_2 and ProstaglandinH_2 by COX. It is central to prostaglandin synthesis and is dependent as an agonist on its conversion, via thromboxane synthase to thromboxane A_2. Thromboxane receptors on the platelet are activated; shape change occurs and release granular products. The COX reaction is the target for aspirin and therefore arachidonate as an agonist is susceptible to analgesic ingestion by the patient.

Epinephrine:

Epinephrine interacts with α_2- adrenergic receptors on the platelet, and only reacts in citrated PRP or in the presence of other agonists. Again, it is inhibited by the ingestion of COX inhibitors.

Collagen:

Collagen is a strong agonist. Fibrils of collagen in suspension adhering to the platelet via glycoprotein (GP) Ia and GPVI cause immediate activation, which initiates release of granule contents and causes a subsequent single-phase pattern. GPIa receptor signals via the phospholipase A_2 pathway, which is inhibited by aspirin, where as GPVI signals via phospholipase C and is not affected. Stimulation of GPIa will also initiate the release of arachidonate with the activation of the phospholipase A_2

Ristocetin:

Ristocetin (Coller & Gralnick, 1977) is product of antibiotic research that was discontinued after thrombocytopaenia was observed in recipients. Ristocetin agglutinates platelets by inducing the binding of von Willebrand Factor (VWF) rather than activating them. This is achieved by phenolic groups on the ristocetin having a

positive charge, causing both a reduction in the overall charge between VWF and GPIb which are both negatively charged. It also causes a structural change in the GPIb to facilitate binding.

1.3.3.2 New Agonists

9, 11 dideoxy-9α-methanoepoxyprostaglandin F2α (U46619):

This stable Thromboxane Mimetic (U46619) (Paul, Jin, & Kunapuli, 1999) 9, 11 dideoxy-9α-methanoepoxyprostaglandin F2α is a thromboxane receptor agonist. It is an analogue of prostaglandin H_2, a prostaglandin that shares a platelet receptor with thromboxane A_2. As has been previously stated, prostaglandins are produced in conjunction with the ADP release from the delta granules. This agonist is useful in determining where a breakdown has occurred. If arachidonate and U46619 fail to aggregate platelets then it is possible that the thromboxane receptor is at fault. If the arachidonate fails, but U46619 activates the PRP then the COX1 conversion of arachidonate to thromboxane mechanism should be under investigation. If the patient has taken COX1 inhibitors then the arachidonate, collagen and epinephrine will be poor agonists, but the mimetic will work.

Calcimycin (A23187):

The calcium ionophore Calcimycin (A23187) or Ionomycin is used in calcium membrane interaction studies. The agonist increases the internal calcium concentration and so activates the platelet. As a large number of the platelet processes are dependent on calcium flux, this is a good method for determining this process rather than detecting it as a secondary affect.

Thrombin Receptor Agonist Peptide (TRAP):

TRAP is used as an alternative to thrombin. A peptide sequence is exposed when thrombin is activated, and this sequence has been synthesised. This compound is used to activate the platelet via the protease-activated receptor 1(PAR-1) without the

interference of other coagulation processes such as the conversion of fibrinogen to fibrin – thus preventing thrombus formation. As it is an artificial specific peptide it gives no information on the interaction of thrombin with the PAR-4 or GPIb receptor.

1.3.4 Secretion assays

Platelet nucleotides, consisting of the adenosine diphosphate and adenosine triphosphate stored in the dense granules, are a useful marker for differentiating and diagnosing dense storage pool disorders.

Initial investigation by platelet aggregation may indicate a storage pool disorder, but is not specific enough to indicate whether alpha granules or dense granules are reduced or absent. Quantification of the contents of the granules, such as ADP and ATP, provides a more diagnostic approach and a relatively simple way of distinguishing the disorders. Those storage pool disorders that involve the dense granules, such as Hermansky-Pudlak and Chediak-Higashi syndromes, have a reduction, or even absence, of their ATP and ADP stores.

Platelet nucleotides were first measured using firefly (*Photinus pyralis*) preparations, which produce luminescence in proportion to the ATP concentration. More recently the active ingredient, luciferase, has been produced artificially and therefore is readily available in kit form.

The luminescence is measured in a luminometer and related to that produced by a standard ATP solution. Phosphoenol pyruvate is added to the reaction to convert the ATP into a measurable stable form in the ATP tube. To measure ADP, pyruvate kinase is added in excess to the reaction causing all of the ADP to be converted into ATP. This reaction is shown in Figure 1.3. This ATP tube is then measured and referred to as ‘total’ and subtracted from the ATP only.

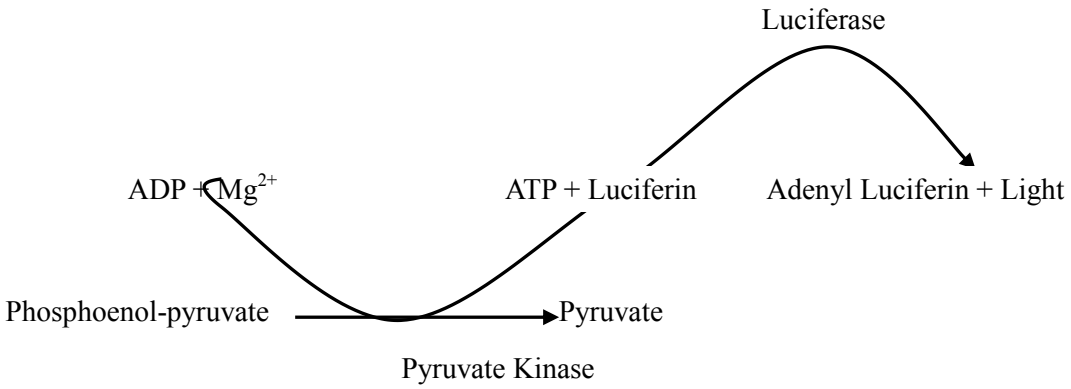

Figure 1.3: Processes used in platelet nucleotide assay

ATP Luciferin-luciferase reagent is a stable light emission (decay rate of ATP and light emission around 0.5%/min). This stability of emission makes it possible to use simple tube luminometers. It is also suitable for ATP measurements down to 10^{-15}mol.

There are alternative analysers using similar methodology to that described above. The Chronolog 700 series whole blood analysers (LabMedics, Stockport, Cheshire) are able to measure platelet aggregation and luminescence on a combined platform. However the Chronolog system only confirms the lack of release. If the Chronolog system and the analysis technique described above are combined then enzymatic disturbances within the platelet can be diagnosed. An example would be a protein kinase defect in which the patients' platelets have normal nucleotide levels however internal enzymatic signalling is defective. The granules are present; they are not being activated and moving to the surface to release their contents.

1.3.5 Flow cytometric assays

Flow cytometers were developed from the automated cell counter which was patented in 1953 by Wallace Coulter. These cell counters used a stream of fluid passing through a charged gate. Each time a cell passed within the gate an electrical impulse would be

created. The size of the impulse denoted the size of the cell. Using size-based guidance, leukocytes being the largest, then erythrocytes, and finally platelets, the smallest these cells could be classed into groups and automated blood cell count could be performed. Using this fine control of particles through a fluid (hydrodynamic focusing) and instead of using charged gates as the detection system, lasers are used. As technology developed the fluid dynamics of the system became more controlled and the underlying electronics improved with micro-circuitry, and this allowed for advanced analysers to be built. A further development was the use of lasers to interrogate cells passing through the gate. The laser was used to evaluate the transparency and shape of the cell, but became further involved after the development of cheaper monoclonal antibodies. These antibodies would attach to receptors of interest and be conjugated with fluorescent dye markers. Depending on the return fluorescence the presence of these specific cell receptors could be evaluated. This has lead to advances in determining different types of leukaemia and increased disease monitoring capability.

This gave rise to the flow cytometer, a phrase that was ratified at 5th American Engineering Foundation Conference on Automated Cytology in Pensacola (Florida) in 1976, just eight years after its invention.

As some platelet disorders are caused by the reduction or mutation in receptors on the platelet surface, these assays methodologies can be used in the diagnosis of these disorders (Michelson, 1996). Receptors such as the GPIIb/IIIa and GPIb on the platelet surface can have monoclonal antibodies raised against them. When these antibodies are conjugated with a fluorescent molecule, it allows rapid diagnosis of Glanzmanns thrombasthenia and Bernard Soulier respectively. Further targets have been sought for platelet analysis (van Velzen et al, 2012) P-selectin (CD62P) and the commercial antibody PAC-1 are only expressed on activated platelets and are used markers for this. Thienopyridine activity can measured using the vasodilator-stimulated phosphoprotein

(VASP) assay (Jakubowski et al., 2008). VASP is an intracellular platelet protein that is non-phosphorylated at rest. Its phosphorylation state is regulated by the cAMP pathway, activated by PGE_1 and deactivated by ADP. The ADP inhibition is mediated by the P_2Y_{12} receptor. The assay involves adding platelets, PGE_1 and ADP and using an antibody against the phosphorylation site of VASP. When the ADP binding to P_2Y_{12} is inhibited by prasgrul or clopidogrel, VASP phosphorylation decreases. Therefore VASP phosphorylation is proportional to P_2Y_{12} inhibition. Expression of Annexin V on the platelet surface is being developed as a diagnostic parameter for Scott syndrome (Flores-Nascimento et al., 2012) and flow methods are also being developed for microparticle analysis (Orozco & Lewis, 2010) and Heparin induced thrombocytopaenia (www.iqproducts.nl/products/specialties/hitalert-kit).

Condition	Platelets		Agonist							Further investigation
	Count	Size	ADP	Coll	Ri	AA	Epi	TRAP	U46619	
Glanzmanns thrombasthenia	N	N	0	0	1	0	0	R/0	R/0	Flow cytometry for GPIIb/IIIa
Bernard-Soulier syndrome	Low	Large	N	N	0	N	N	N	N	Flow cytometry for GPIb
COX Deficiency	N	N	1/R	R	N	R	R	N	N	COX Analysis
Drug induced (aspirin shown)	N	N	1/R	R	N	R/0	R	N	N	Stop aspirin (use of questionnaire)
Thromboxane receptor defects	N	N	1/N	R	N	R	N	N	R	Platelet nucleotide/ biochemistry analysis
$P2Y_{12}$ receptor defects	N	N	R/0	N	N	N	N	N	N	Receptor analysis/ biochemistry analysis
Ehlers Danlo syndrome	N	N	N	N	N	N	N	N	N	Genetic testing Other physical manifestations
VWD	N	N	N	N	0/R/N	N	N	N	N	VWF screening
Storage Pool defects										
Grey platelet syndrome	Low	N	R/1	N/R	N	N	N	R	N	Electron microscopy
Quebec platelet syndrome	N	N	N	N	N	0	N	N	N	Multimerin analysis
Chediak-Higashi syndrome	N	N	R/1	R	N	N	R	N	N	Dense granule/nucleotide analysis
Hermansky Pudlak syndrome	N	N	R/1	R	N	N	R	N	N	Dense granule/nucleotide analysis Other physical manifestations
Membrane anomalies										
Scott syndrome	N	N	N	*N*	N	N	N	N	N	Flow cytometric Annexin V analysis Genetic testing
Paris-trousseau syndrome	Low	Large	N/R	*N*	N	N	N	R/1	N	Electron microscopy for large alpha-granules. Other physical manifestations

Table 1.2: Describing major platelet function disorders and their manifestation in aggregation studies with additional laboratory follow up. Modified from Moore & Gurney, 2010

Key: N=normal, 0=absent, 1=primary wave only, R=reduced, Coll=collagen, Ri=ristocetin, AA=arachidonic acid, Epi=epinephrine, COX=cyclo-oxygenase, TRAP=thrombin receptor agonist peptide

1.4 Diagnostic pathways

Bristol is at the junction of three main areas of the UK: the south west peninsula (Dorset, Devon and Cornwall), Wales (Vale of Glamorgan and surrounds), and western counties (Wiltshire and Oxfordshire), and is positioned to provide a significant region with specialist services. There are also strong collaborative ties with the University of Bristol's departments of pharmacology and biochemistry.

Platelets function disorders are a heterogeneous group of common bleeding disorders that require specialist technical and clinical expertise. The fact that there are many disorders with differing treatment protocols depending on the diagnosis gives rise to the need for a specialist laboratory.

Prior to 2006 at Bristol platelet testing procedures were lacking in three main areas:

- Systematic and comprehensive testing repertoire driven by evidence based practice
- Specialist techniques to support routine testing
- The skills and experience in reporting specialist tests

Recently a number of diagnostic algorithms have become available-most notably the UK review of Bolton Maggs et al (Bolton-Maggs et al., 2006) that highlighted deficiencies in the diagnostic pathway(s)

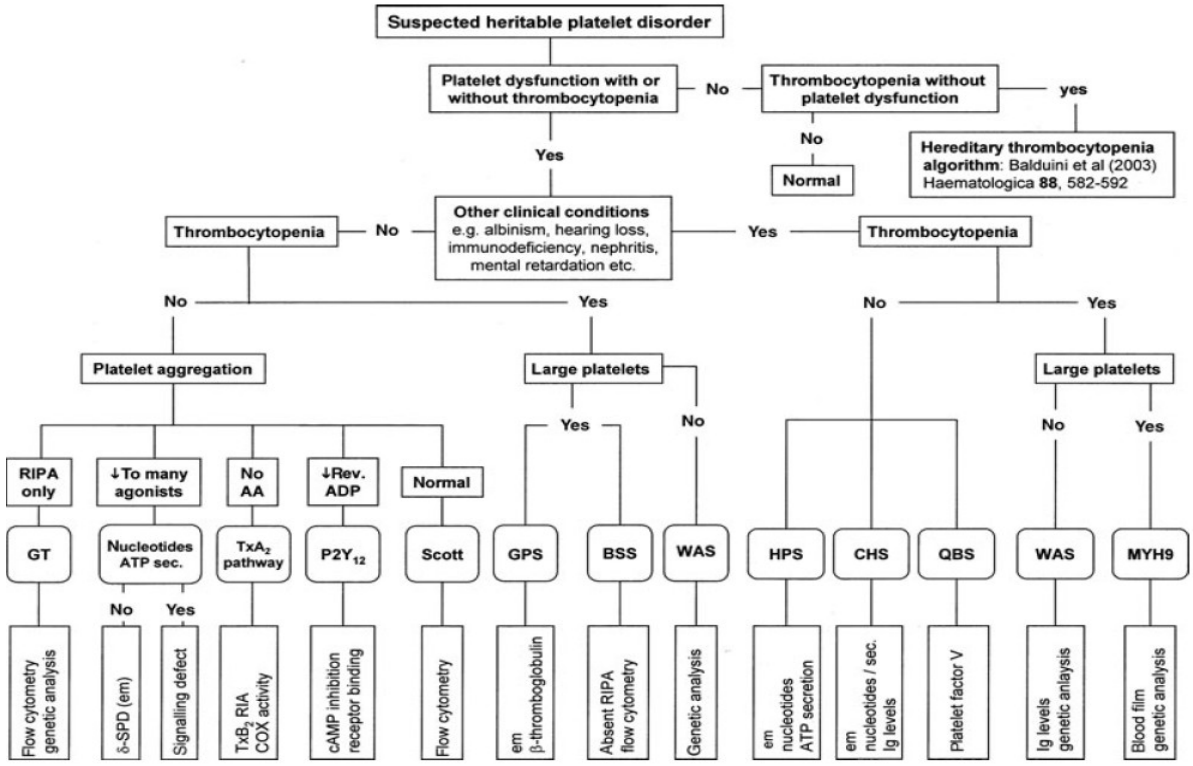

Figure 1.4: Diagnostic algorithm from Bolton Maggs et al

It was felt that as a regional centre Bristol could not fulfil all the diagnostic areas required of a comprehensive laboratory service. Platelet aggregation was not developed and therefore there could be a delay in diagnosis of 43% of the disorders listed in the figure above. Furthermore, platelet nucleotide and glycoprotein analysis were out sourced to other specialist laboratories, leading to a delay for a 36% of diagnoses. This led to unacceptable turnaround times for results to be available to the treating clinician; on occasion the return of results could take three months. This was deemed unacceptable and there was therefore a need for testing to be brought in house. Particularly important was glycoprotein analysis, as it could be performed on small samples. This would form part of the urgent paediatric platelet screen. In bringing the platelet aggregation to a first line test and the glycoproteins and release assays in-house there is now the facility to increase diagnostic work (according to the figure above) by over 50%.

No test is ever foolproof and a skilled specialist scientist is needed to assist in selection, performing and interpretation of results to support the clinical team in obtaining the correct diagnosis for the patient.

1.5 Aims and Objectives

The active involvement of platelets in the haemostatic process demands that platelet analysis is an integral part of patient investigation methodologies in the haemostasis laboratory's repertoire. It has been shown above that the methods for assessing platelet numbers, function and contents are numerous and are not without their limitations. Those methods that the laboratory chooses to employ should be `fit for purpose'. This can only be achieved by thorough method assessment, complemented by knowledge of the strengths and weaknesses of each assay.

Therefore the overall objectives of this project are to develop platelet laboratory protocols that will enhance the assessment of platelet function and improve diagnostic accuracy in a cost-effective manner. To support this main objective it is intended that the following developments, procedures and evaluations will be incorporated:

1. Minimisation of pre-analytical variability in platelet function testing, using questionnaires, standardised sample collection and clinical interview.
2. Improvement in standardisation of light transmission platelet aggregometry (LTA) by generating new reference ranges for the common agonists ADP, Ristocetin, Collagen, Epinephrine and Arachidonate.
3. Addition to the LTA repertoire of – and generation of reference ranges for – two new agonists: a thrombin receptor agonist peptide (TRAP) and a stable thromboxane mimetic U46619.
4. Comparison of two sources of equine tendon collagen in order to determine whether

a more cost-effective preparation is comparable to that recommended in the guidelines as the ‘gold standard’ (i.e.: with the aim to reduce the cost of the assay without sacrificing quality).

5. Standardisation of platelet nucleotide reference ranges.
6. Implementation of a flow cytometric assay for platelet glycoprotein analysis, to include selection of antibodies and construction of reference intervals.

In the general discussion I will provide evidence of:

7. Integrating all of the above into a specialist laboratory environment encompassing a comprehensive, streamlined process for the diagnosis of platelet function disorder.
8. Achieving recognition of this specialist service, not only locally and regionally, but ultimately nationally and internationally.

Chapter 2: Materials and Methods

2.1 Standardisation and minimisation of pre-analytical variables

Standardisation of the pre-analytical variables is a complex task to achieve. By its very definition it happens before the sample is analysed and therefore part of it does not come under the auspices of the trained laboratory personnel. Therefore, to standardise the procedures one has to engage many staff groups.

The medical staff at Bristol Royal Infirmary are aware of standardised questionnaires for interviewing patients with more common bleeding disorders such as haemophilia (Rodeghiero et al., 2010; Rydz & James, 2012) and there have been moves to modify this for inherited platelet disorders (Marcus et al, 2010). Clinical consultations involve detailed questioning regarding drugs and diet as mentioned above.

2.1.1 Pre-analytical Questionnaire

To reinforce the clinical consultation and assist the nursing staff, and to make available a laboratory document, a pre-analytical questionnaire was devised (Gurney, 2004). The questionnaire was initially devised by interrogating the papers referenced earlier with respect to diet, lifestyle and pharmaceutical intake.

Discussion took place between the haemophilia centre nursing staff, haemophilia clinical team, and relevant laboratory personnel to ensure that the questionnaire was fully understood and therefore able to be used to maximum effect. Nursing staff were made aware that a questionnaire was required to be completed with every request for platelet aggregation testing. The questionnaire was also used to inform the scientist writing the final report.

This questionnaire has been through various versions depending on needs of the particular laboratory operating it. The original version was initially devised at Guys and St. Thomas hospitals, and revisions were subsequently made for its use at BRI

2.1.2 Sample collection standardisation

Additional information was included with the questionnaire, in order to standardise sample collection, based on recent International Society for Thrombosis and Haemostasis guidelines (Harrison et al., 2011). This procedural advice included: veni-puncture procedure; use of a 'discard' tube (to minimise the amount of tissue fluid in the resulting sample); anticoagulant choice; and time limits on collection to testing period. To facilitate the latter a member of the laboratory staff collects the samples from the clinic. This not only speeds up transport of samples, it ensures the time of sampling is known and prevents the samples being transported via a pneumatic delivery system (Glas et al,, 2012)

2.2 Generation of reference ranges for Light Transmission Aggregation

To achieve standardisation of LTA in our laboratory more efficiently and cost-effectively the decision was taken to upgrade the current platelet aggregometer from the current Biodata PAP-4D to the Biodata PAP-8E (AlphaLabs, Eastleigh, Hants).
The introduction of the ISTH recommended panel (Harrison et al., 2011) has also necessitated generating new reference ranges for the common agonists: ADP, ristocetin, collagen, epinephrine and arachidonate. These recent ISTH guidelines also recommended the addition of two new agonists: TRAP and a stable thromboxane mimetic U46619. These have been also been introduced to the laboratories' testing repertoire. Therefore this section details the procedures for generating reference ranges for the expanded range of agonists.

2.2.1 Subjects

Reference controls used to produce reference ranges were volunteers from within the laboratory and associated staff. The reference ranges were developed by collating the data produced from thirty four references samples (16 males, 18 females; mean age 38) during routine platelet aggregation studies.

All individuals were initially screened using the pre-analytical questionnaire.

2.2.2 Sample collection and Preparation

Samples were taken and transported to the laboratory as described above (see 2.1). They were then centrifuged at 200g for 10 minutes with no brake applied at the end of centrifugation. The resulting platelet-rich plasma (PRP) was transferred from the sample to a plastic tube using a plastic pasteur pipette, taking care to avoid the buffy coat. The PRP samples were then capped and stored at 20-25^{0}C whilst the original samples were re-centrifuged at 3000g for ten minutes to produce platelet-poor plasma (PPP).

2.2.3 Materials

All agonists (identified above) were supplied by Hart Biologicals (Hart Biologicals, Hartlepool, County Durham). Dilutions of each agonist were prepared as shown in table 2.1 below and stored at 20-25 degrees until added.

Agonist	Reconstitution Fluid	Stock Solution	Working Solution	Final concentration in PRP.
ADP	Reagent grade water	200μM	**1)** 150μL **stock** + 150μL Saline (100μM) **2)** 50μL **(1)** + 50μL Saline **3)** 50μL **(1)** + 200μL Saline **4)** 50μL **(3)** +100μL Saline	25μL agonist + 225μL PRP **1)** 10 μM **2)** 5 μM **3)** 2 μM **4)** 1 μM
Collagen (Nycomed)	Buffer supplied in kit pH 2.8	1 mg/mL	**1)**50μL **stock** +950μL kit buffer **2)**50μL **(1)** +200 μL kit buffer	25μL agonist + 225μL PRP **1)** 10μg/mL **2)** 2μg /mL
Ristocetin	Physiological saline	20 g/L	1) Neat stock 2) 50μL (1) + 50μL Saline	25μl agonist + 225μl PRP 1.25 g/L 0.6 g/L
Arachidonate	Reagent grade water	5mg/mL	Use neat (50μL)	1mM 25μL agonist + 225μl PRP
Epinephrine	Reagent grade water	1 mM	**1)** 50μL **stock** +200μL Saline **2)** 50μL **(1)** + 450μL Saline	25μl agonist + 225μl PRP **1)** 20 μ M **2)** 2 μ M
Thrombin Receptor Agonist Peptide (TRAP)	Reagent grade water	568μL in vial 1.73mg/mL 1mM	**1)** 150μL (2) + 150μL Saline **2)** 100μL (3) + 900μL Saline	25μL agonist + 225μL PRP **3)** 10μM **4)** 1μM
U-46619	Physiological saline	500μM	**1)** Neat **2)** 100μL(3) + 900μL Saline	25μl agonist + 225μl PRP **3)** 5μM **4)** 1μM

Table 2.1: Details of the dilutions and final concentrations of the agonists used in the platelet aggregation assays

2.2.4 Method

Light transmission was measured through a stirred sample, taking the PPP as the 100% reference and the PRP as 0%. The agonist was added directly to the PRP, the analyser determined the increase in light transmission caused by platelets aggregating and produced a trace. The reaction was allowed to continue for at least five minutes. In order to assess the distribution of normal aggregation procedure, traces were run using the highest dose of each agonist and preparing a trace graph with eight different controls.

2.3 Comparison of two collagen sources for light transmission aggregometry

2.3.1 Subjects

25 subjects (13 Males 12 Females with an average age 38 years) were assayed. This was a sub population of the group in 2.2.1.

2.3.2 Sample collection and preparation

Samples were collected as described and prepared as described in 2.2.2.

2.3.3 Materials

Horm collagen preparation has been the gold standard preparation for a number of years (Yardumian, Mackie, & Machin, 1986). It is manufactured by Nycomed and prepared from equine tendon collagen particles derived from collagen sponge strips. The method consists of several grinding and mixing procedures finally resulting in a suspension that contains 1 mg/ml of native collagen fibrils in pH 2.7 – 2.9 glucose buffer solution, with parahydroxibenzoicacid –methylester and

parahydroxibenzoicacid –propylylester preservatives. Most of the alternative collagen preparations on the market are of bovine origin and would therefore not be suitable as a direct comparison. Hart Biologicals were the first reagent company to supply an alternative equine tendon preparation. The reagent supplied by Hart was produced in a very similar way but the suspension medium had less biocides. The lack of biocides gives it a shorter shelf life, but should not interfere with the assay.

Horm collagen and the alternative source were supplied by ABP pharmaceuticals (ABP, New Jersey, USA).

2.3.4 Method

Each subject had platelet aggregation performed on a PAP-8E analyser as described in 2.2. For this study both collagen preparations were run in parallel, using 10µg/mL and 2 µg/mL dilutions. Maximum aggregation, initial slope and lag phase data points were recorded from the analyser.

2.4 Generation of a Platelet nucleotide assay reference range

Platelet nucleotides, consisting of the ADP and ATP stored in the dense granules, are a useful marker for differentiating and diagnosing dense storage pool disorders. Assays used to assess nucleotide levels can be broadly separated into two groups; release during aggregation, and absolute quantification. Quantification of nucleotides enables diagnosis of delta storage pool disorders (SPD) and distinguishing from synthetic defect in release cycle. More recently the active ingredient, luciferase, has been produced artificially and therefore is readily available in kit form.

2.4.1 Subjects

Data was collected from 31 control subjects (21 female and 10 male, average age 32 range 18-52)

2.4.2 Sample collection

Platelet nucleotide analysis was performed on PRP, collected and prepared as described above (see 2.2.2). A platelet count was performed on an automated cell counter and the PRP adjusted to 300 x 10^9/L with hard spun platelet poor plasma (PPP). Once the PRP was prepared 500µL was aliquoted into a small container. To this was added 50µL 0.134M EDTA to chelate the calcium. The mixture was then vigorously mixed. Into this mixture 50µL 20% Triton X-100 was added. This breaks down the platelet membrane and releases the cytosol. After further vigorous mixing and 500µL Absolute Ethanol was added which precipitates out the membrane solids and leaves the cellular fluid for analysis. The sample was mixed again and left to stand at 4°C for 15-30 minutes; after this the samples were stored at -70°C, (where they are stable for up to 6 months).

2.4.3 Materials

The luminometer used in the laboratory was in need of replacing. Luminometers were therefore selected for comparison based on their capacity to perform to the criteria of our assay. These criteria included the ability to output raw data in both printed form and to a computer in a recognised format. The ability to construct the standard curves and then relate subsequent samples and controls to the curve was considered desirable, but not essential as the necessary spreadsheets for calculating values from raw data were already in use in the laboratory.

Luminometers that were requested for in-house evaluation were the Berthold LB9507 (Jencons) and the Luminoskan (Thermo). Both instruments met the brief,

but it was decided on a cost basis that only the Berthold LB9507 would be evaluated against the current method.

Luminescent reagents (ATP standard and ATP monitoring reagent) were all manufactured by Biotherma (Biotherma, Handen, Sweden) and supplied by LabTech International (LabTech International, East Sussex, UK)

2.4.4 Method

An ATP standard curve was generated using a known ATP standard. An ADP control was incubated with pyruvate kinase and incubated for 15 minutes. The control was analysed on the luminometer to confirm the ADP to ATP reaction had taken place. Once completed the confirmation was checked using the following equation; luminescence of the ADP control divided by the luminescence top point on the standard curve multiplied by one hundred (to give a percentage conversion).

If the percentage conversion was less that 80% then the assay was restarted. Once the conversion was confirmed the samples were defrosted, centrifuged and the supernatant aliquoted into testing vials. The supernatant phosphoenol pyruvate was added in excess and assayed to determine the ATP concentration. An additional aliquot of the original supernatant/mixture was then mixed with pyruvate kinase. The reaction of phosphoenol pyruvate to pyruvate, catalysed by pyruvate kinase, is driven by ADP and ATP. The ATP value of the original sample is referred to as initial ATP and the pyruvate catalysed ATP as total ATP. To calculate the ADP, initial ATP is subtracted from the total ATP.

2.5 Generation of a flow cytometric glycoprotein reference range

2.5.1 Subjects

Data was collected from 32 adult control subjects (26 female and 6 male, average age 40 range 16-72) and 11 paediatric control subjects (6 male and 5 female, average age 1.5years range, 1day -2years).

2.5.2 Sample collection

The adult samples were taken using the same phlebotomy procedure as for platelet aggregation (see 2.2.2). The platelet aggregation control sample was used for data acquisition and if the aggregation on the patient was normal that was used also.

Of the paediatric samples, seven were collected as samples coming to the laboratory for routine analysis of glycoproteins, and when no abnormality was found they were included in the reference pool. Three samples were collected after verbal consent was obtained, as umbilical cord samples, in collaboration with midwives at St Michaels Hospital, Bristol.

2.5.3 Materials

2.5.3.1 Antibody selection

To establish an enumeration technique for these important receptors, the appropriate antibodies were first selected. To select the antibodies a number of manufacturers were approached, who produced the antibodies of interest (in this case anti GP IIb/IIIa), and asked to participate in the trial. Three manufacturers participated: AbD Serotec (Endeavour House, Langford Lane, Kidlington, OX5 1GE, UK);Beckman Coulter (Oakley Court, Kingsmead Business Park, London Road, High Wycombe, HP11 1JU); and R&D Systems (Abingdon, Oxford UK).

Company	Clone	Isotype	Conjugate	PE/ Ig ratio	Catalogue Number	Lot Number	Recommended Concentration
Serotec	Y2/ 51	IgG1	PE	not stated	MCA2588PE	0307	Not stated. Lyophilised. Recommend add 1ml, use 10ul per test. Probably 100ug/ml
Serotec	PM6/13	IgG1	PE	not stated	MCA728PE	0304	Not stated. Lyophilized. Recommend add 1ml, use 10ul per test. Probably 100ug/ml
Coulter	SZ21	IgG1	PE	0.5-1.5	PN IM3605	16	Not stated. 20ul per test of 500,000 cells
R&D Systems	256809	IgG2a	PE	not stated	FAB2266P	LNP 0209101	10ug/ml

Table 2.2: Characteristics and sources of antibodies considered.

2.5.3.2 Optimisation of antibody

The analyser was set up and checked for noise and background fluorescence. The antibodies were then diluted to ascertain their optimal dilution and fluorescence stability. The graphs below illustrate this data for the R&D and Beckman Coulter antibodies. The Coulter antibody maintained fluorescence at increased dilutions. It is also demonstrated more linear fluorescence. This antibody was therefore selected for further study.

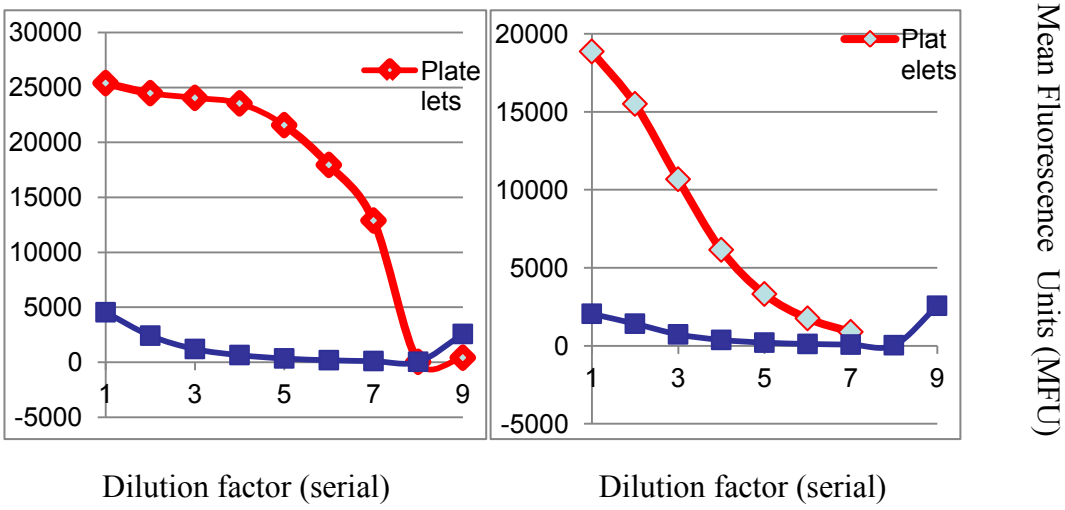

Figure 2.1: Dilution and stability graphs

Graphs showing the dilution and stability of two manufacturers antibodies. The Coulter antibody is represented in the graph on the right, the R&D antibody in the graph on the left. The Coulter antibody has a more stable fluorescence at a higher dilution.

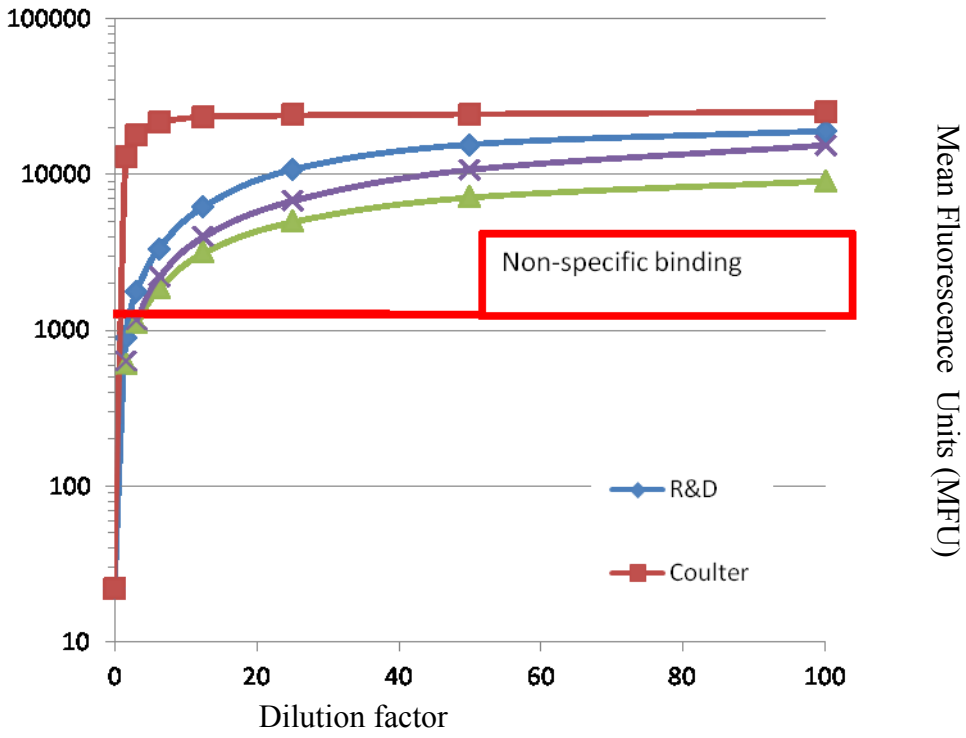

Figure 2.2: Mean fluorescence vs. dilution for all antibodies demonstrating the additional range of the Coulter antibody

2.5.4 Methods

The samples were diluted one in twenty with Becton Dickinson FACSFlow© (Becton Dickinson, Oxford, UK) reagent, a saline based transport fluid for Becton Dickinson flow cytometers. The antibody was diluted one in ten with FACSFlow©. 80µL of antibody solution and 10µL of the diluted blood were then incubated, at room temperature, for 15 minutes in the dark. After incubation 200µL of FACSFlow© was added to stop the reaction. These samples were then processed using a Becton Dickinson LSR© flow cytometer (Becton Dickinson, Oxford, UK), reading at least 5000 events (Schmitz et al., 1998) which had been calibrated previously using rainbow and cytometer setup and tracking (CST) beads.

Chapter 3: Results

3.0 Results

3.1 Pre-analytical questionnaire

The variability in presentation of platelet function disorder requires a standard procedure followed to minimise interfering factors. To do this a pre-analytical questionnaire was implemented at Bristol in July 2010 (See table 3.3 St Thomas' and 3.4a-c Bristol). Since 2010, when the new questionnaire became part of the testing process, there have been 85 platelet aggregation assays performed. Of these there were 22 (26%) patients identified that had ingested substances that could affect the results of the assay. Sixteen (19%) were taking prescription medication and six (7%) taking over the counter (OTC) or herbal preparations. Six (27%) affected patients assays needed repeating after the subjects had abstained from the `identified substances' for at least fortnight. The other 16 were reported as having no abnormality observed, with the caveat that it had been noted the patient had been taking drugs known to interfere with platelet function. The two case studies described below are used as illustrations:

Questionnaire for all platelet aggregation patients & controls

Question	Response
Are you taking?	
• Aspirin (within the last 14 days MINIMUM)	
• OTC Cold Relief Medication e.g. Lemsip	
• Ibuprofen	
On Any of the Prescription Medication below? COX1 inhibitors (indomethicin, sulfinpyrazone, naproxen) ADP receptor agonists,(ticlopidine, clopidogrel) GPIIb/IIIa agonists, (Rheopro, tirofiban, eptitifabatide, Intelligrin) Prostaglandins (Cliostazol), Tri-cyclic antidepressants (Imipramine, amitripyline)	
If the PATIENT has answered YES to the above please reschedule. If the CONTROL has answered YES please find another volunteer.	
On any other current prescription medication? Please circle relevant drugs. Penicillin, ampicillin, propranolol, atenolol, captopril, perindopril, anaesthesia,	
Are you currently taking vitamin supplements? **If yes tick or circle if listed below** Vitamins B6, C, E all affect platelets	
Are you taking dietary supplements or herbal remedies? **If yes please tick or circle if listed below:** Starflower oil, Fish oil, Ginko Biloba and Green Tea all have platelet antagonistic ingredients	
Have you had a take-away within the last 48 hours? Elements in Turmeric, Cumin, Onion, Garlic, Ginger, Clove, black tree fungus & mono sodium glutamate all affect platelets.	
Do you drink or smoke? Alcohol, Caffeine & Smoking have been known to affect platelets	
Was this a clean Draw?	

Figure 3.1: Initial questionnaire developed at St Thomas hospital

LTA PLATELET STUDY

For first-line investigation of platelet bleeding disorders

Surname	
Forename	
UH Bristol or NHS No	
Date of birth	

First Line investigation?	
Follow Up investigation?	
Family study? (please identify proband)	

Document exposure to any of the following in the last 2 weeks	
Aspirin	
'Over the counter' cold relief medication	
Ibuprofen	
Any of the Prescription Medication below? NSAIDs: Indomethicin, Sulfinpyrazone, Naproxen ADP blocker: Ticlopidine, Clopidogrel, Prasugel GPIIb/IIIa agonists: Reopro, Tirofiban, Epitifibatide, Intelligrin Prostaglandins: Cliostazol Antidepressants: Citalopram, seroxat imipramine, amitripyline	
List all prescription drugs below	
List all vitamin or dietary supplements and herbal remedies in last 10 days **e.g.** Starflower oil, Fish oil, Ginko Biloba and Green Tea Vitamins B6, C, E	
Alcohol, caffeine or tobacco in last 24 hours?	

Figure 3.2a: Current extended questionnaire used at

Bristol Royal Infirmary Part a

Control	
Initials	
Gender	
Age	
Expose to anti-platelet agents in last 10 days (y/n)	

- Please contact Mr David Gurney, Senior BMS (or designee) on ext 22598 before blood sample is taken. Please report exposure to anti-platelet drugs - inpatient or control.

- Collect one 'purple top' EDTA sample, followed by 6-8 4.5ml 'blue top' citrate samples, using a needle gauge between 19 and 21 if appropriate. Use minimal venous stasis. Please discard tubes that are incompletely filled or in which the blood draw has been difficult.

- The specimens may also be suitable for factor and VWF assays. Please indicate on separate request form/ICE request. Please enclose ICE request stickers or paper form with this request.

Specimen collection	
Collection date	
Collection time	
Traumatic venepuncture?	

Figure 3.2b: Current extended questionnaire used at

Bristol Royal Infirmary Part b

To be completed by the laboratory:

- Prepare PRP from citrate samples 15 min at 170g followed by PPP
- Process EDTA sample for blood count, mean platelet volume and film.

Time of sample receipt	
Platelet count on EDTA sample	
Platelet count on PRP preparation	
Appearance of PPP	**Clear / Lipaemic / Haemolysed**

- Only if PRP count > $^{600\times109}$ per litre, dilute down to 600 using PPP

Count on PRP after adjustment	

Start time of aggregation study	
End time of aggregation study	

Report:
Report compiled by: **Date:**

Figure 3.2c: Current extended questionnaire used at Bristol Royal Infirmary Part c

Case Study One: A subject with unexplained peri-operative bleeding was referred to the haematology clinic for investigation. Standard haemostasis testing (coagulation screen, factor VIII, factor IX and von Willebrand screening) was performed and these were all normal. At this point in the diagnostic process the clinician requested a platelet aggregation analysis. Upon filling in the questionnaire the patient informed the nurse that they were taking multivitamins. When subject to further questioning the patient admitted to taking allium (garlic) capsules, calcium citrate with vitamin D, quercetin with bromelain, cod liver oil, co-codamol, fluconazole, cetirizine, and high dose vitamin C. None of these were listed in his pre-operative clinical history. As noted in section 1.2.2 many of these items may interfere with platelet aggregation. A reduced ADP response (shown as lines 1-4 in Figure 3.3), compared to the control was observed, and it was decided to repeat the assay after stopping these products. Repeat testing demonstrated no untoward results (shown as lines 5-8 in Figure 3.3). Without the questionnaire and the nursing staff diligence this patient could have been diagnosed as mild platelet function defect of unknown determination.

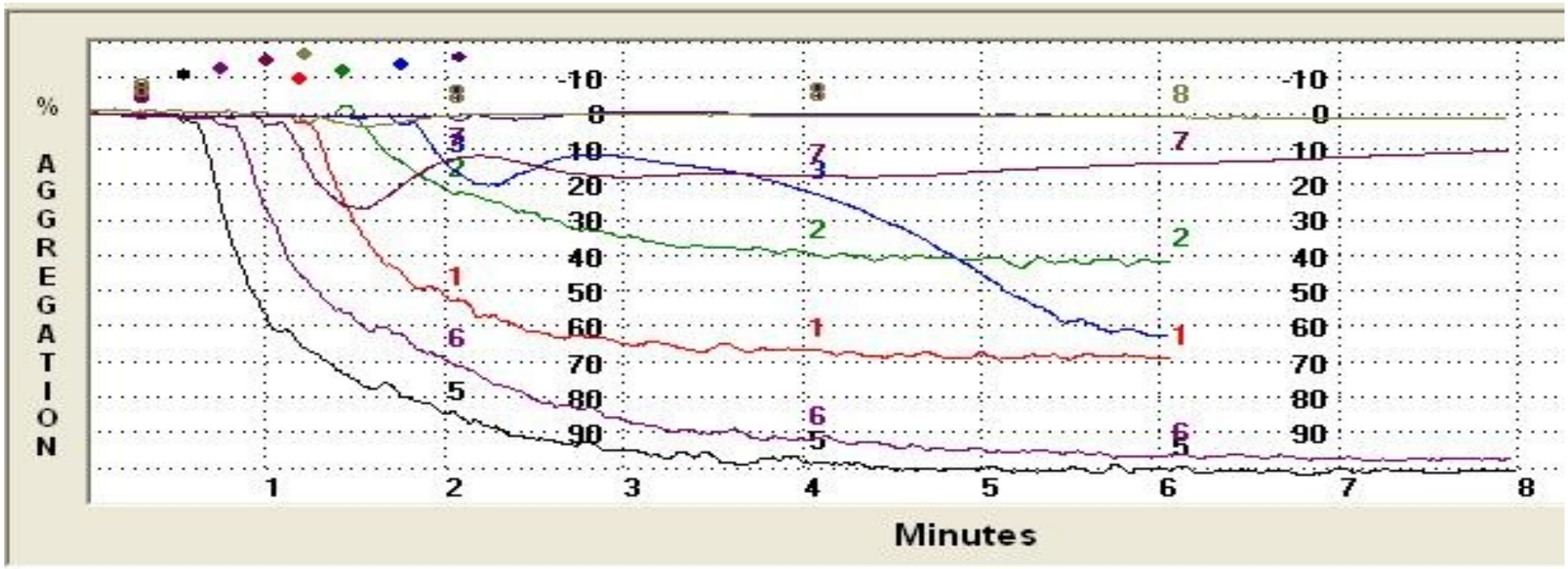

Figure 3.3: ADP aggregometer traces on patient SL:

Pre: Line 1 10µM ADP, Line 2 5µM ADP, Line 3 2µM ADP, Line 4 1µM ADP

Post: Line 5 10µM ADP, Line 6 5µM ADP, Line 7 2µM ADP, Line 8 1µM ADP

Case Study Two: On completing the pre-analytical questionnaire a second patient, BF, was found to have been prescribed propantheline bromide for excessive sweating. On further investigation it was discovered that this patient had also been investigated by the paediatric team some 18 months previously. This meant we had access to pre drug, on drug and off drug data.

Propantheline belongs to the anti-muscarinics which down regulates acetylcholine receptors. As these receptors are G-coupled receptors there was concern that they also down regulate ADP receptors as these are also G-coupled. In Figure 3.4 lines 1 and 2 show the patients' response to low dose ADP before the patient was prescribed the drug, lines 3 and 4 show the patients' response whilst on the drug and lines 5 and 6 when the patient was retested after abstaining from the drug for a month. It was demonstrated that this patient had a mild bleeding defect as yet uncharacterised that seem to be up-regulated whilst on the medication.

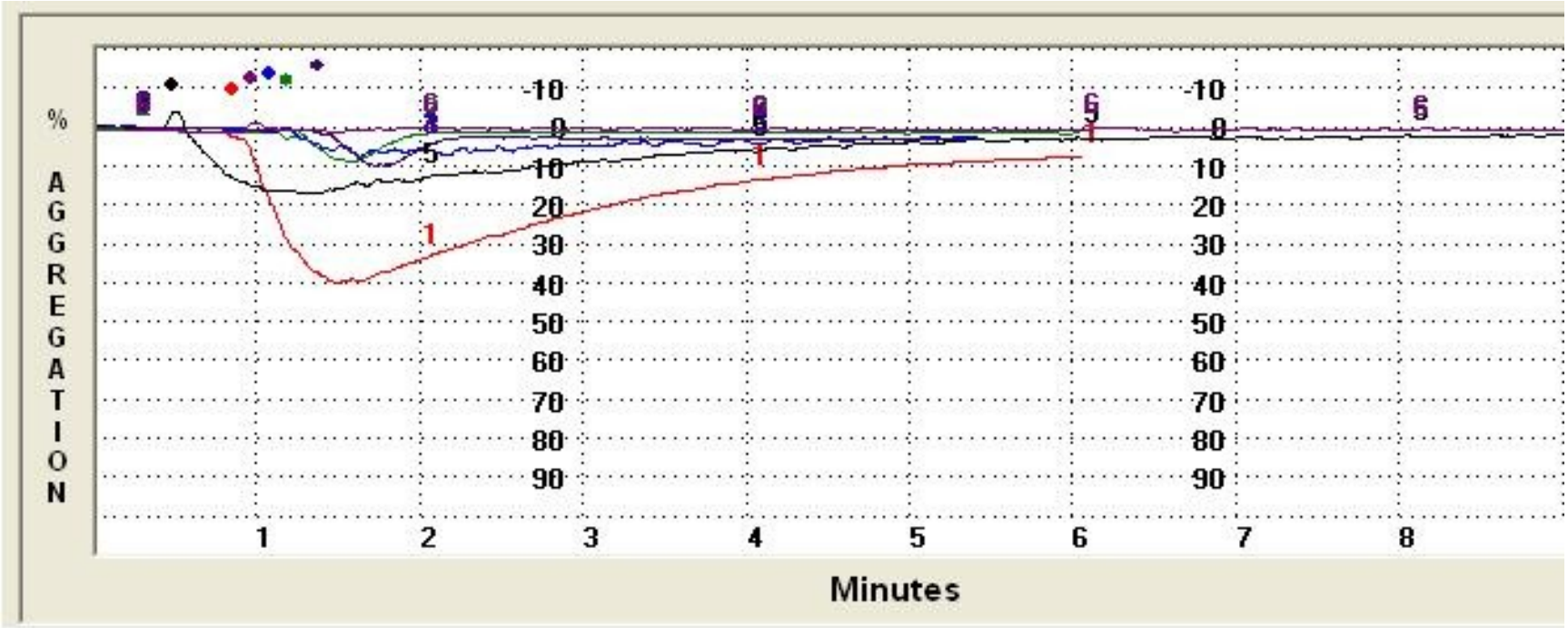

Figure 3.4: ADP aggregometer traces for BF:

March 2011 Line 1 2µM ADP, Line 2 1µM ADP,

July 2012 Line 3 2µM ADP, Line 4 1µM ADP

August 2012 Line 5 2µM ADP, Line 6 1µM ADP

3.2 Generation of reference range data for light transmission aggregation

A series of graphs giving a visual representation of the data obtained for the platelet agonist references ranges is shown. The scatter plots in Figure 3.5 illustrate that there is minimal lag in the ADP at 10μM and 5 μM and the initial slope values are more spread as the concentrations decrease. This is most noticeable in the 2 plots, as it is at this point that the platelets can be induced to produce a biphasic response. This concludes with either a second phase (release) or a full reversal (disaggregation) of the agglutination process. On the scatter plot below (Figure 3.6), detailing the results for collagen, ristocetin and epinephrine, it is demonstrated that the collagen reaction happens in uniform groups were the epinephrine has a wide range of results at 20μM and two discrete groups at 2μM. Variation in response of normal controls to epinephrine is well known (Harrison et al., 2011)where the epinephrine has a wide range of results at 20μM and two discrete groups at 2μM. In the reference group for low dose ristocetin (0.6m/L) there are some noticeable responders which could be mild hyper-responders. This is normally indicative of Type 2B VWD or pseudo/platelet type VWD and should be investigated further. There are two subjects who respond to low dose ristocetin ~100%. As indicated this is indicative of Type 2B VWD or pseudo/platelet type VWD and these candidates should have ristocetin induced mixing studies or genetic analysis to determine if there is a disorder present. There is a group of six subjects whose aggregation with low dose ristocetin ~75%. This is increased and there is an argument for removing these patients from the reference range as they are not considered ‘normal’. However this then becomes an artificial construct, if

these patients are exhibiting no symptoms, should not the definition of 'normal' be altered? The answer to this theoretical argument is to increase the sample population to establish the percentage of these outliers.

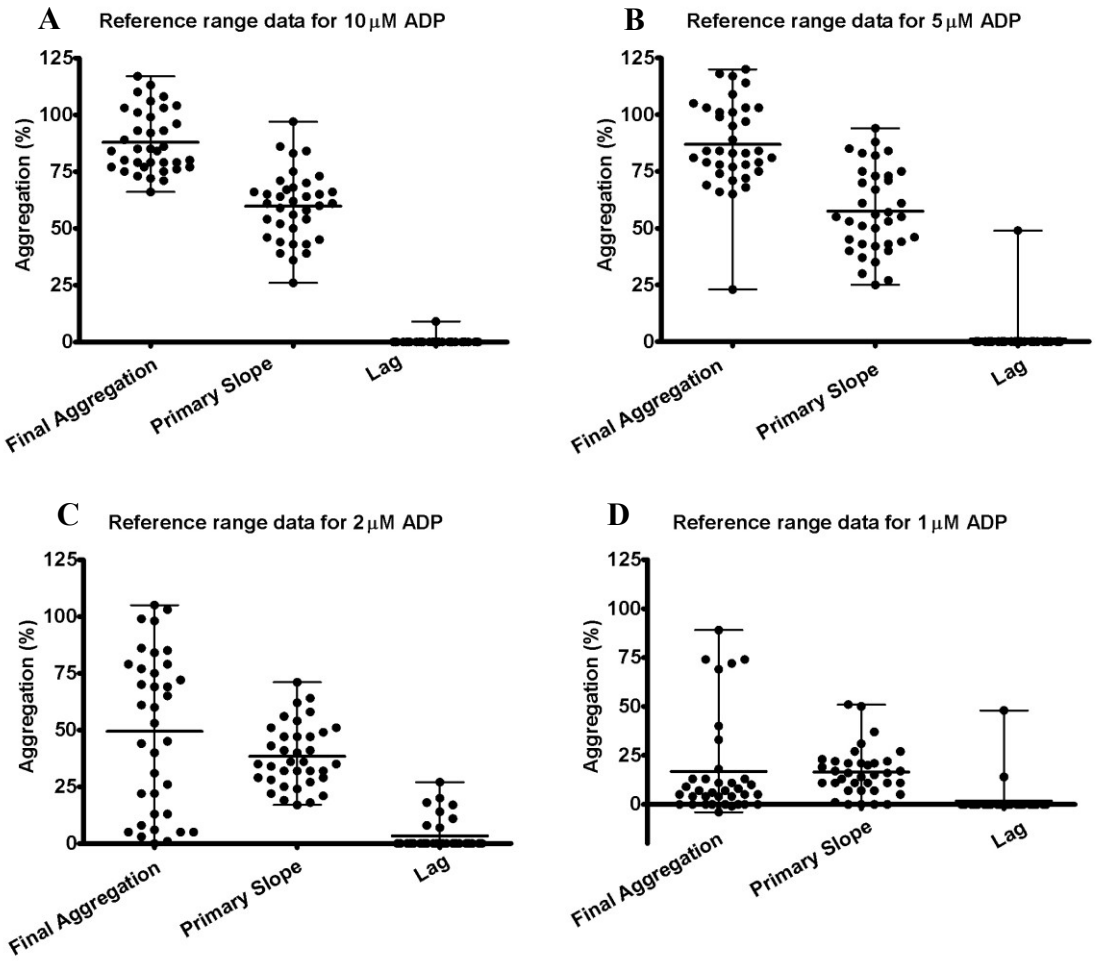

Figure 3.5: Scatter plots for ADP reference range

(A) shows maximum aggregation, slope and lag for 10μM ADP, **(B)** shows maximum aggregation, slope and lag for 5μM ADP, **(C)** shows maximum aggregation, slope and lag for 2μM ADP, and **(D)** shows maximum aggregation, slope and lag for 1μM ADP,

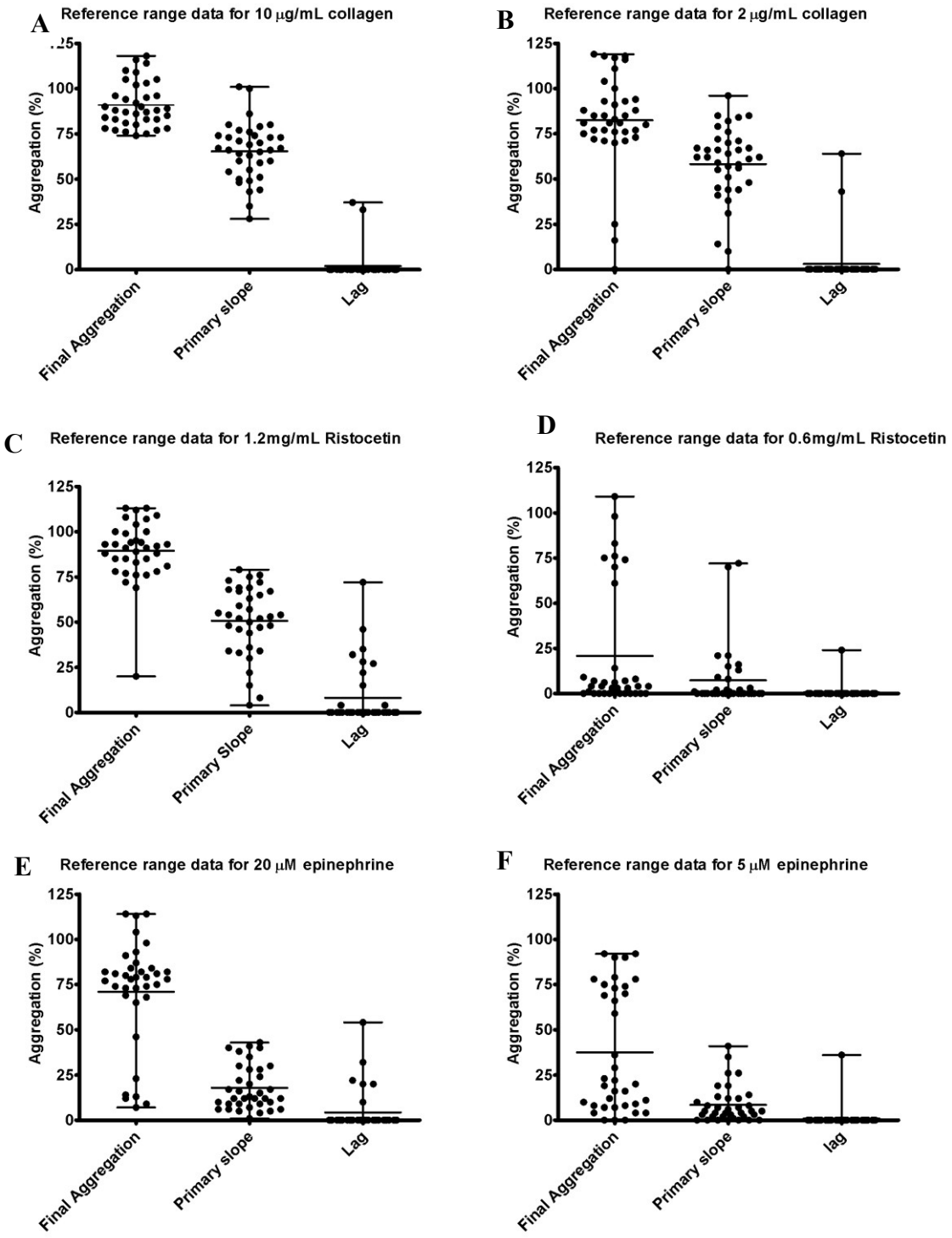

Figure 3.6: Scatter plots detailing range for final aggregation, primary slope, and lag data for all concentrations of collagen (top), **ristocetin** (middle) **and epinephrine** (bottom)

(A) Shows final aggregation, primary slope and lag for 10μg/mL collagen (B) Shows final aggregation, primary slope and lag for 2μg/mL collagen (C) Shows final aggregation, primary slope and lag for 1.2mg/mL ristocetin (D) Shows final aggregation, primary slope and lag for 0.6 mg/mL ristocetin (E) Shows final aggregation, primary slope and lag for 20μM epinephrine (F) Shows final aggregation, primary slope and lag for 20μM epinephrine

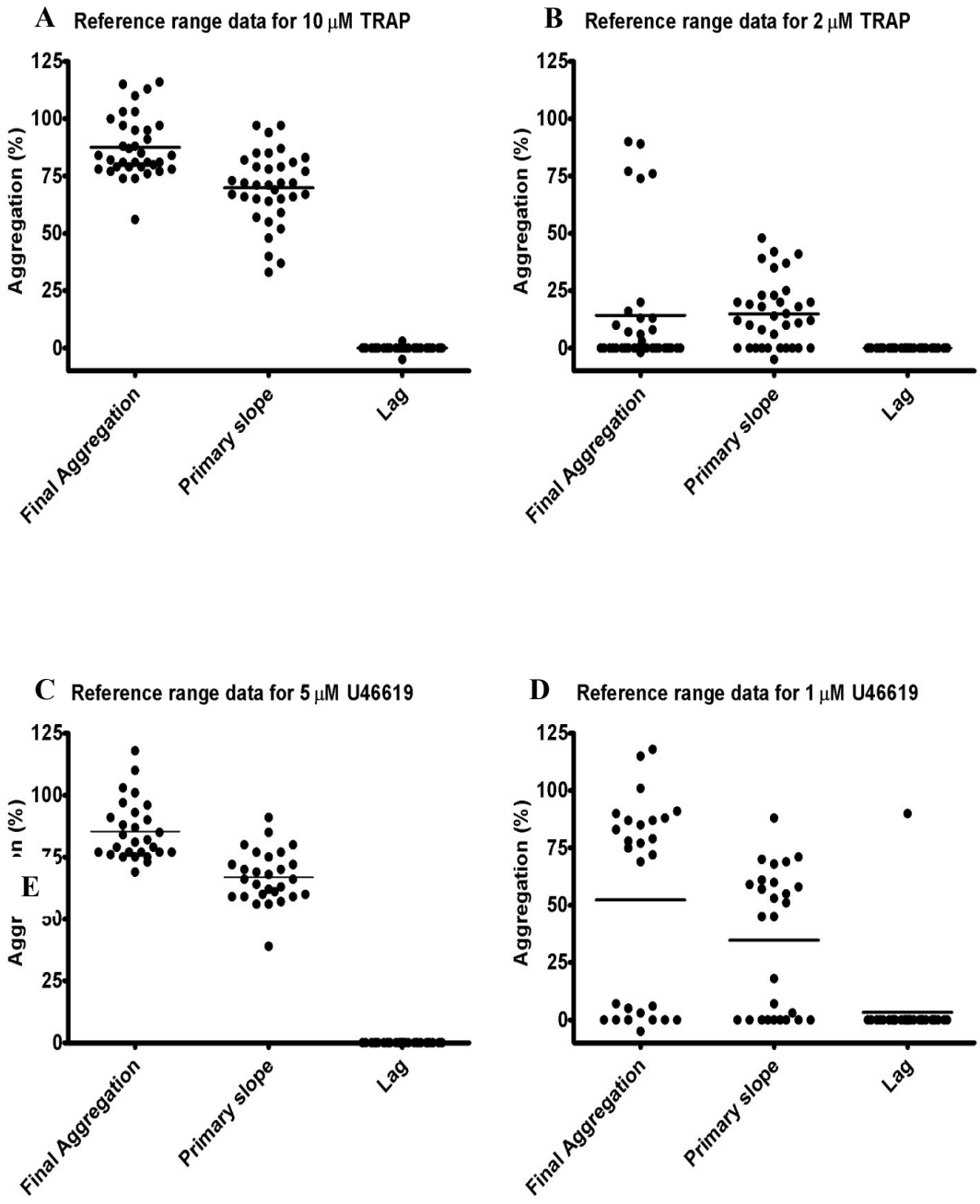

Figure 3.7: Scatter plots for TRAP and U46619 reference range

(A) shows maximum aggregation, slope and lag at 10μM TRAP, **(B)** shows maximum aggregation, slope and lag at 2μM TRAP, **(C)** shows maximum aggregation, slope and lag at 5μM U46619, and **(D)** shows maximum aggregation, slope and lag at 1μM U46619

Agonist	Concentration	Slope		Maximum aggregation		Lag phase	
		Mean (Median)	2SD	Mean (Median)	2SD	Mean (Median)	2SD
ADP	10µM	60 (61)	30	88 (84)	28	0 (0)	4
	5µM	57 (65)	36	87 (84)	38	1 (0)	16
	2µM	38 (35)	28	50 (53)	68	3 (0)	14
	1µM	16 (15)	24	14 (13)	22	2 (0)	16
Collagen	10µg/mL	65 (66)	32	91 (88)	24	2 (0)	16
	2µg/mL	58 (62)	42	82 (84)	52	3 (0)	24
Ristocetin	1.2g/L	51 (53)	38	89 (91)	34	8 (0)	32
	0.6gL	7 (0)	34	20 (4)	66	1 (0)	8
Epinephrine	20µM	18 (13)	24	71 (79)	58	4 (0)	22
	2µM	8 (5)	20	36 (20)	66	1 (0)	12
TRAP	10µM	69 (71)	32	88 (84)	26	0 (0)	2
	1µM	15 (12)	30	16 (10)	38	0 (0)	0
U46619	5µM	67 (66)	118	86 (83)	22	0 (0)	0
	1µM	35 (45)	62	52 (75)	88	3 (0)	34
Arachidonate	1.6mM	54(57)	40	84(83)	42	7(0)	22

Table 3.1: Details of the mean (median) and twice standard deviation (SD) for all agonists at working dilutions.

dilutions for major platelet aggregation parameters giving the reference range (Mean +/- 2SD)

It has been assumed that the data is parametric; this is supported by the fact that the mean and median are similar for most data points. It is noticeable (highlighted points on table 3.1) that where there is variation between the mean and median these are at low concentrations of agonists and there is no uniform skew. (ristocetin being skewed to the right and U46619 being skewed to the left). The reference range is calculated as mean +/- 2 standard deviations from the mean. This takes in 95% of the data population.. The scatter plots in Figure 3.7 illustrate the dose response nature of the agonist TRAP. However the scatter plots for U46619 demonstrate that at lower doses the populations are split into two discrete groups. Both final aggregation responses and primary slope have separated into groups. There are no documented receptor polymorphisms which would explain this result. However the concentration of the agonist is at a critical threshold and the data demonstrates that a small shift to the right on a steep dose response curve has a dramatic response.

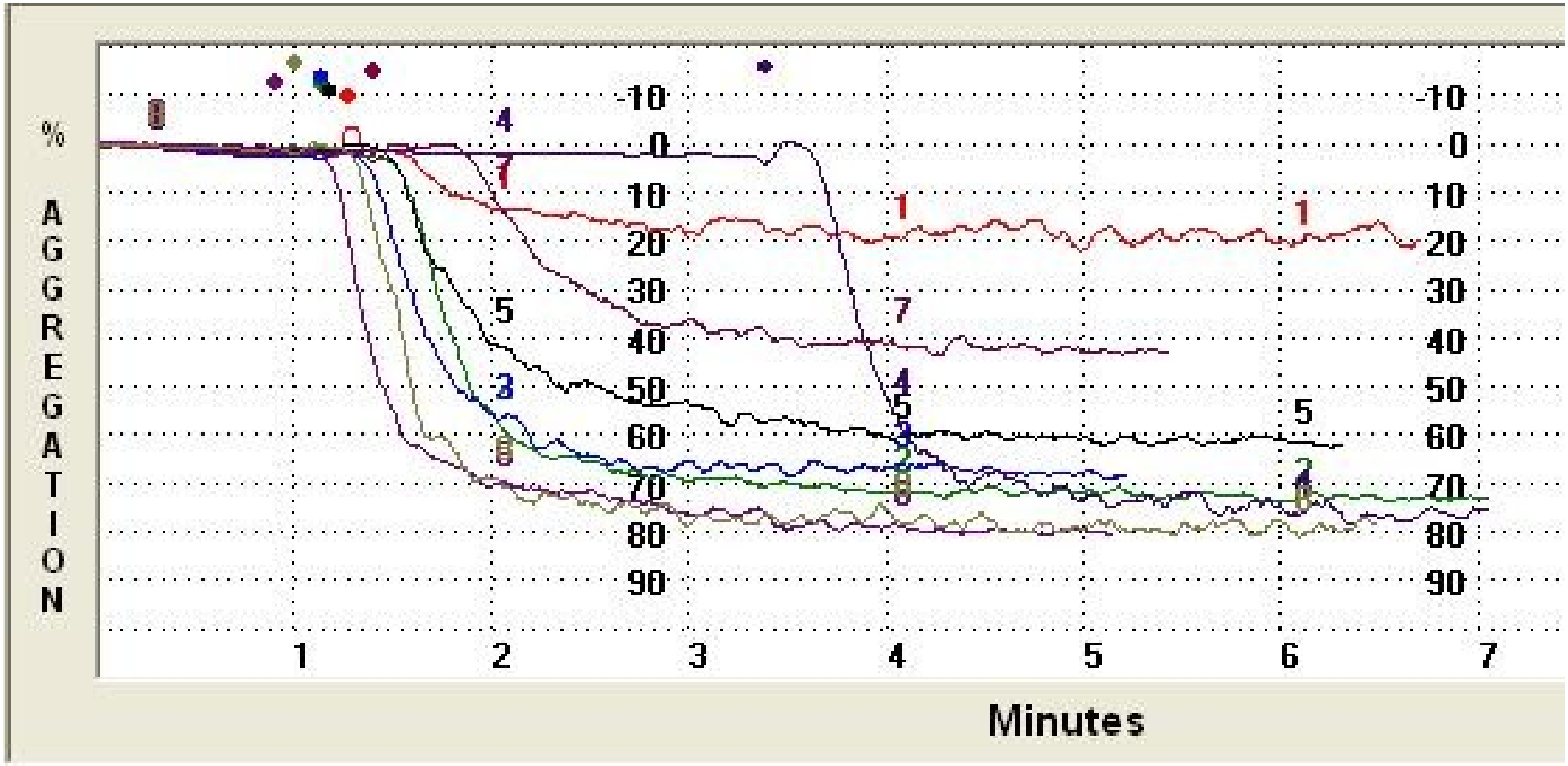

Figure 3.8: Showing PAP-8E traces for multiple control subjects at 10μM ADP

Figure 3.8 on the previous page illustrates the wide ranges noted in the table on the previous page. As a qualitative assay the reference intervals are wide. The different lag phases (especially in line 4 Figure 3.8) and the slope and maximal aggregation (Lines 1 & 7 Figure 3.8) of all with the same dose ADP.

3.3 Results for collagen reagent comparison.

Collagen is a key agonist in the platelet aggregation stimulating the GPVI receptor. However the current reagent is expensive. It was proposed to compare the current reagent with a reagent new to the market but with a substantially lower price. The object was to critically compare the preparations in healthy control population. Maximum aggregation, slope and lag phase data points, were taken from the PAP-8E analyser and statistically analysed using GraphPad Prism (La Jolla, California, USA). Scatter plots, correlation curves, and Bland Altman plots shown below were produced (Figures 3.9–3.11). These show that using a high dose (10µg/mL) agonist there is little difference in results for maximum aggregation between the two preparations of collagen. Horm collagen has a mean of 90% with a median of 87% compared to Hart 87% and 82% respectively. The absolute ranges (that is minimum value to the maximum value) is 72-116% for Horm and 70-116% for Hart. This pattern is repeated with the slope and lag values. With the slope data Horm mean is 69', median 68' (absolute range 49'-93'), with the Hart reagent it is 67, 66 (51-91) respectively and lag data Horm mean 22s, median 19s (0-34s) compared to the Hart reagent mean 22s, median

21s (0-51s). This data is shown on the two left hand data clusters on the scatter graphs in Figure 3.9. On the right hand side of these cluster graphs is demonstrated the wider variation at the lower doses of collagen.

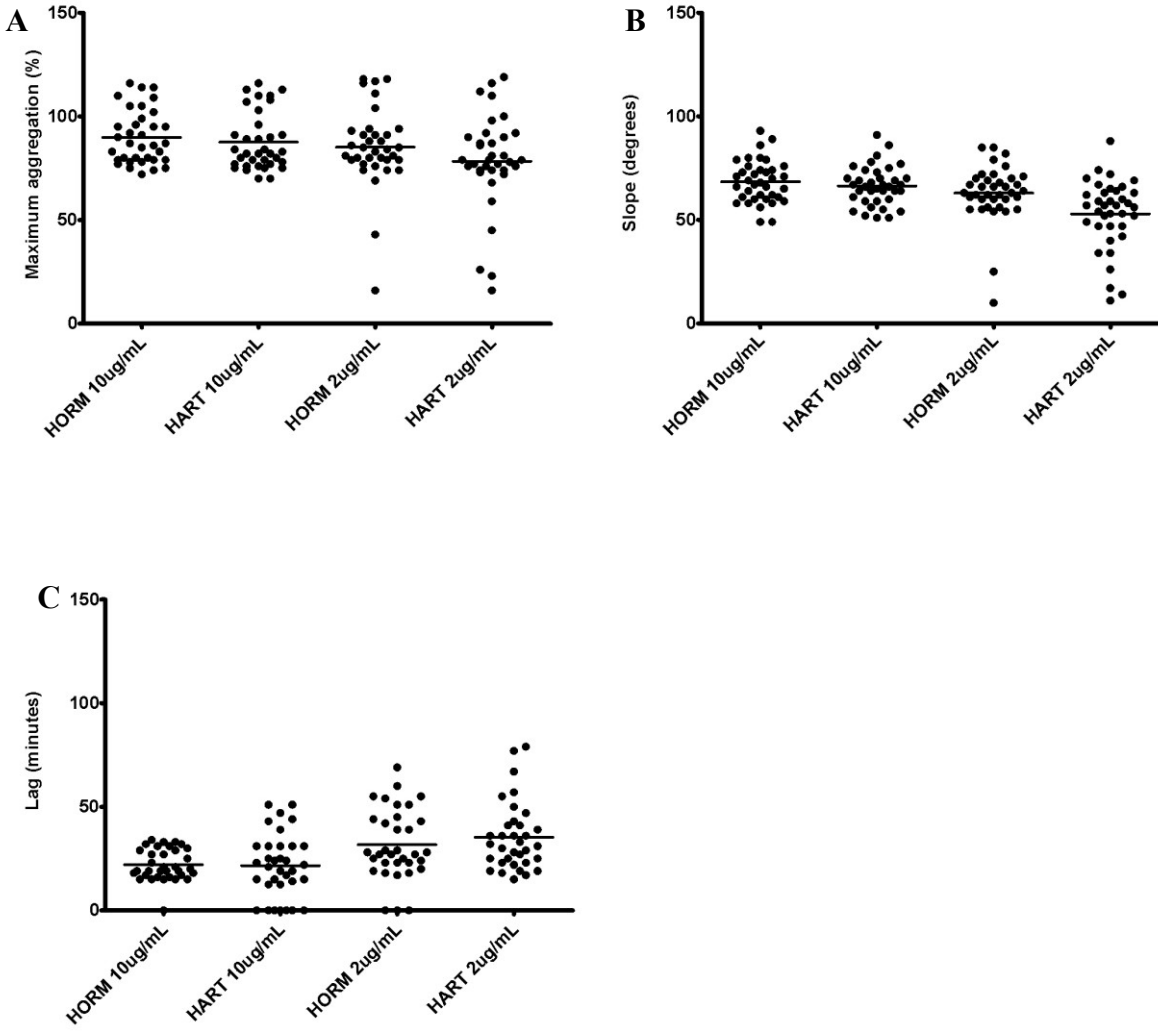

Figure 3.9: Scatter graphs of comparison data

Maximum aggregation **(A)**, slope **(B)** and lag **(C)** using 10µg/mL and 2µg/mL Horm and Hart collagen

Linear regression analysis was performed and the graphs can be seen below in figure 3.10. Figure 3.10a is maximum aggregation with high dose collagen on the

left and low dose on the right. As observed with the scatter there is good agreement for maximum aggregation with high dose collagen (r^2 0.92, $p<0.001$), however it is much poorer for low dose collagen (r^2 0.77, $p<0.001$) At 2mg/mL collagen the Hart reagent produces lower values for both maximum aggregation and slope, as is demonstrated by the two points parallel with the X axis on the maximum aggregation graph and three points on the slope graph. For the lag phase and the slope correlation was poor at both collagen concentrations (lag phase 10µg/mL r^2 0.36, $p<0.001$ and slope 10µg/mL r^2 0.41, $p<0.001$). This data is summarised in table 3.2.

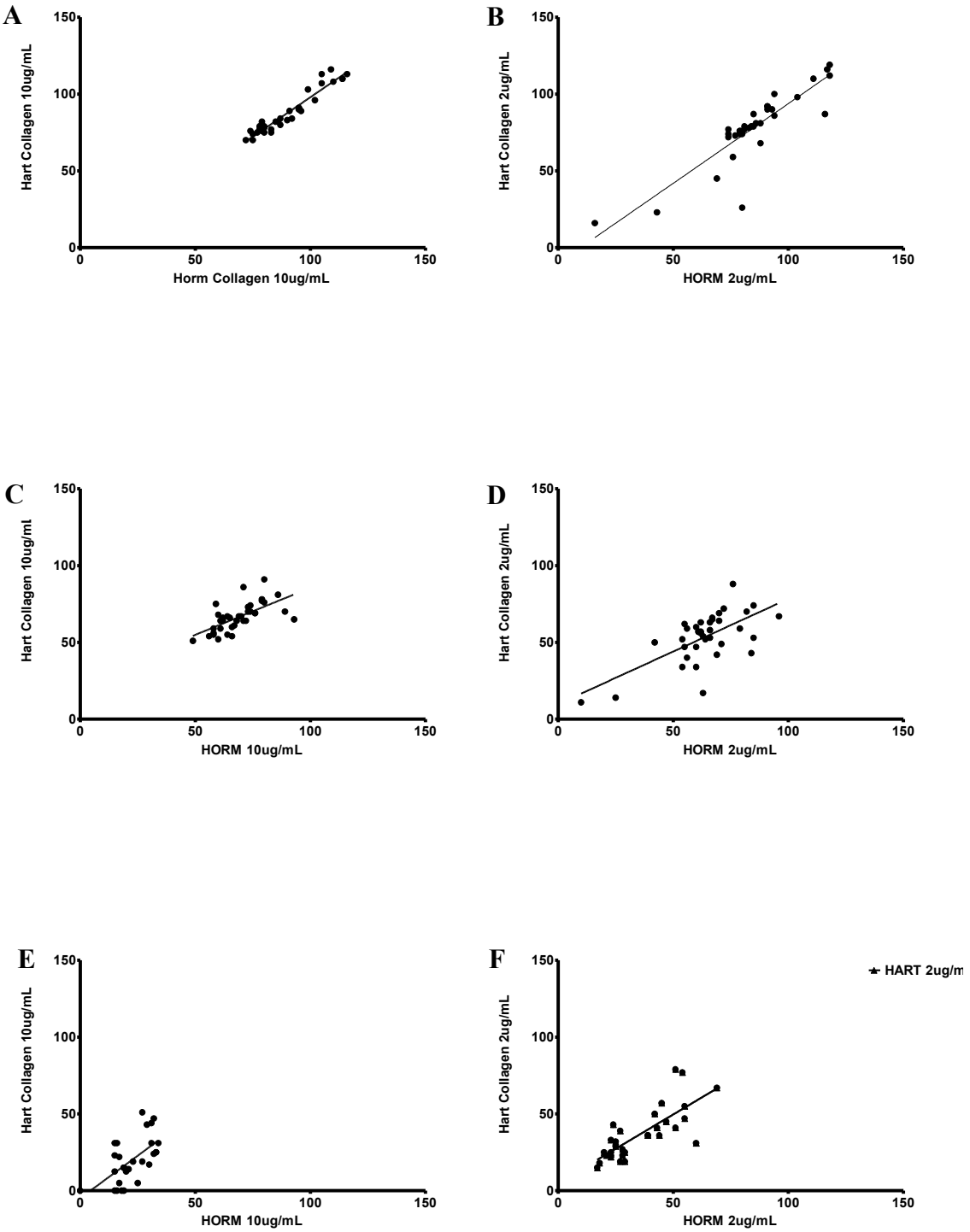

Figure 3.10: Linear regression analysis of comparison data

Maximum aggregation (**A** and **B**) slope (**C** and **D**) and lag phase (**E** and **F**). 10µg/mL collagen is depicted in the left hand column and 2mg/mL in the right hand column

The Bland Altman plots below for the maximum aggregation at high dose collagen (Figure 3.11 top left) show good agreement with minimal bias. At low agonist dose, reflecting the outliers on linear regression curve the maximum aggregation, there is a positive bias and broader spread (with one point above the upper limit of agreement) on the Bland Altman plot (Figure 3.11 top right). However for slope (Figure 3.11 middle plots) and lag phase (Figure 3.11 bottom) there is wide variation at both concentrations of agonists with little correlation on the regression curves. The Bland Altman plots show a wide spread with the slope having a positive bias and the lag phase has negative bias.

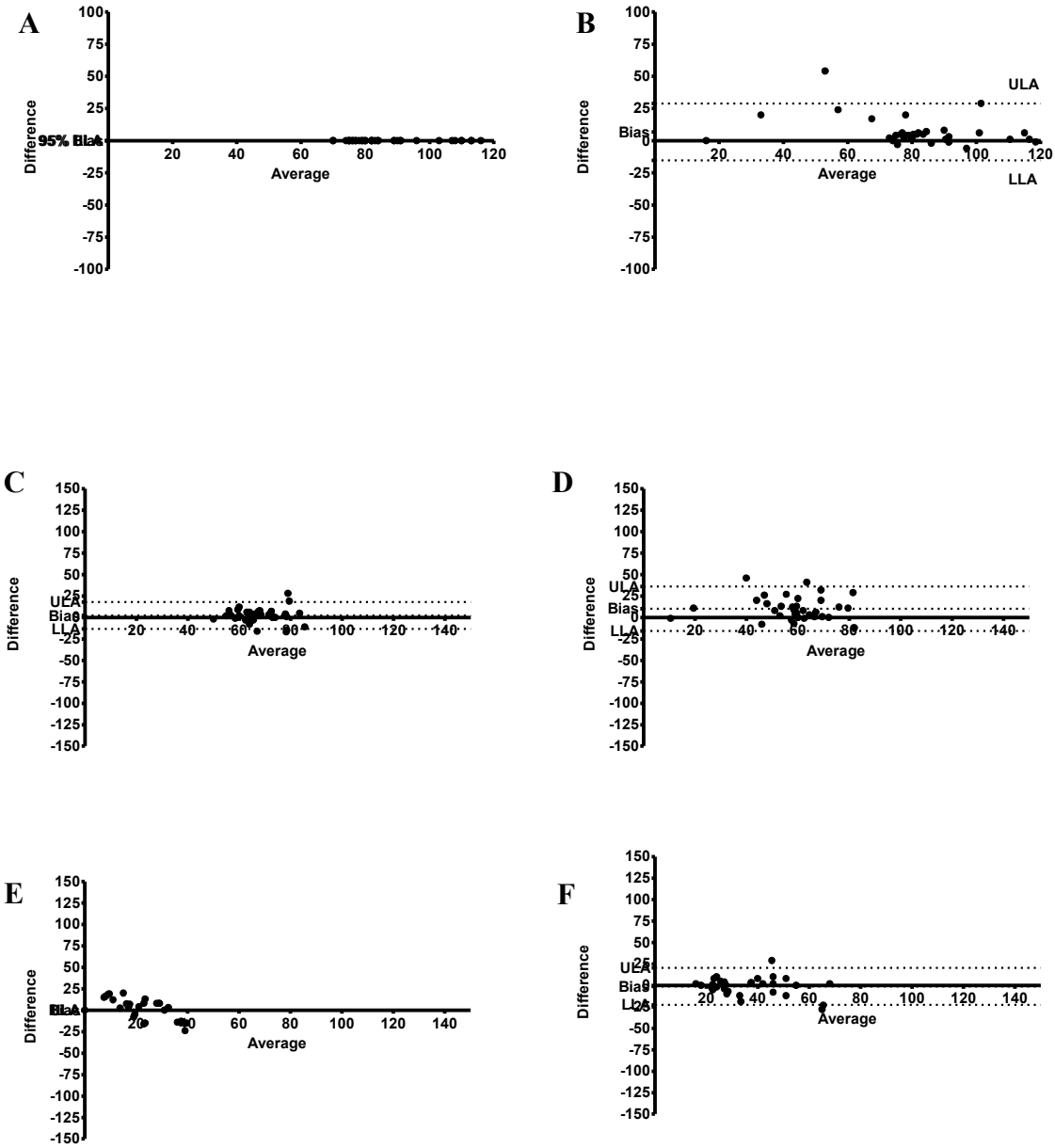

Figure 3.11: Bland Altman analysis of comparison data

Maximum aggregation (**A** and **B**) slope (**C** and **D**) and lag phase (**E** and **F**). 10µg/mL collagen is depicted in the left hand column and 2µg/mL in the right hand column

	Collagen concentration (µg/mL)	r^2	p	Confidence Interval (95%)
Maximum Aggregation	10	0.92	<0.001	0.9 – 1.1
	2	0.77	<0.001	0.8 – 1.2
Lag Phase	10	0.36	0.006	0.5 – 1.7
	2	0.58	<0.001	0.6 – 1.2
Slope	10	0.41	<0.001	0.4 – 0.9
	2	0.43	<0.001	0.4 – 0.9

Table 3.2: Table showing statistical values for comparison between Horm and Hart collagen preparations

In summary at 10µg/mL final concentration of collagen there is good grouping with little significant difference between the reagents. By contrast at 2µg/mL there is a significant difference. When plotted, the regression for the maximum aggregation is good, but less so for slope and lag phase. At 2mg/mL there is poor correlation overall all parameters. When the maximum aggregation was plotted the Bland Altman show good agreement at 10mg/mL Agreement was poorer with slope and lag phase. At 2µg/mL there is poor agreement across all parameters.

3.4 Generation of a platelet nucleotide reference range g

Platelet nucleotide analysis is essential for establishing the diagnosis of a delta storage pool (dense granule) disorder. New equipment had been purchased to improve reliability. This necessitated the establishment of a new reference range for the new equipment and that data is presented below. In figure 3.12 the measured values of ATP and total ATP, and calculated values of ADP, adjusted ATP and adjusted totals a are given with mean and ranges. Where the PRP count was less than the required 300 or could not be adjusted physically with PPP a mathematical adjustment was made to ensure correlation. Adjustment was made by dividing the desired platelet count by the actual platelet count of the PRP. In figure 3.12 below the mean adjusted ATP is 17, with a range of 7-43. This range is higher than the unadjusted ATP 15 (7-27). This is due to a low platelet count causing an outlier to be amplified. (e.g. PRP count of 100 x 10^9/L would cause a multiplication adjustment factor of 3) This adjustment factor and resulting high value for ATP would also explain the negative value for ADP on the scatter plot. If both the total adjusted ATP and the adjusted residual ATP are artificially raised by a lower platelet count, then a low or negative value would be the result. This then becomes the lower limit of the assays sensitivity. In addition low platelet counts will amplify analytical error. In a normal platelet dense granule there is twice the ADP than ATP (Mills & Thomas, 1969) the mean on the scatter plot is 2.8, the reported median is 1.3. Dividing the amount of nucleotide by the platelet count in the PRP (Figure 3.12 right hand side) The data is summarised in table 3.3

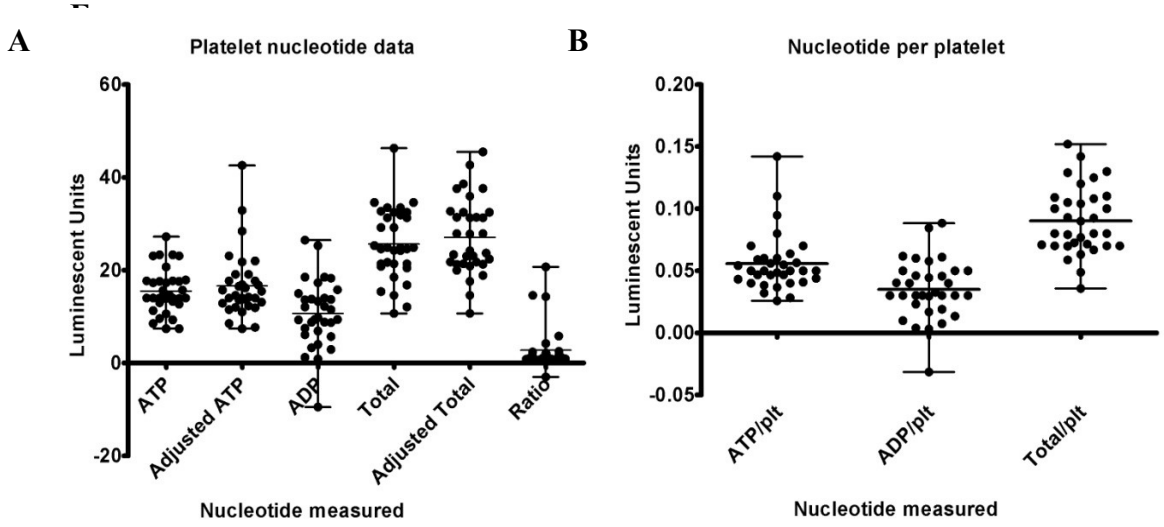

Figure 3.12: Scatter plots showing data for platelet nucleotide reference ranges

Plot **A** shows ranges for platelet nucleotides and the plot **B** nucleotide/platelet ratio

	Mean (Median)	SD	(95%)
Platelet Count	290 (300)	55	271-309
ATP	17 (15)	7.1	14-19
ADP	11 (10)	7.1	8-13
Total	27 (25)	7.7	24-30
Ratio	2.8	4.7	1.1-4.4

Table 3.3: Reference range values for platelet nucleotide analysis.

The data in this table shows the ranges when constructed using mean +/- 2SD

The given reference range for platelet counts on automated analysers is normally given in the range of 150 - 400 x 10^9/L. In our study the range is 180 - 400 x 10^9/L which is comparable and indicates that a representative sample of the 'normal' population has been sampled. The mean and the median results show good agreement and so the data is assumed to be parametric. Therefore the reference

range for this data was calculated as mean +/- 2SD. Using this calculation the ADP reference interval is 0-26 fluorescent light units (FLU). However the confidence interval indicates that 95% limits are 8-13 FLU. This is more plausible as a patient with no ADP in the dense granules would not be considered 'normal'. The confidence interval assumes knowledge of a value for the wider population being calculated, where as the calculated reference interval is based only on the sample population. An alternative is to use the median +/- 2SEM. Standard error of the mean (SEM) involves the mean in the calculation so the true value is known, where as standard deviation is just scatter from the mean. Using SEM includes the mean in the calculation; the spread is smaller for small populations and becomes larger in larger populations. Larger populations assume that you are nearer to the 'actual' mean. Given this the nucleotide reference intervals would be calculated as shown in table 3.4 below.

	Median	2SEM	CI (95%)
Platelet Count	300	19	271-309
ATP	15	2.4	14-19
ADP	10	2.6	8-13
Total	25	2.8	24-30
Ratio	2.8	4.7	1.1-4.4

Table 3.4: Showing reference intervals for platelet nucleotides

The reference range is calculated as Median +/- 2SEM

Using median +/- 2SEM, the reference range for the platelet count becomes of 281-319 x 10^9/L, which is a smaller give range than the 95% confidence interval. This would be unusable in a modern haematology laboratory as it is too

constrictive. However the nucleotide reference intervals now compare well with the 95% confidence intervals and are more realistic.

3.5 Generation of a platelet glycoprotein reference range

Bringing an assay in-house requires the same reference range work up as establishing a new assay. Control samples for platelet aggregation and patients that exhibited no abnormal reaction in the platelet aggregation assay were enrolled into the glycoprotein reference range study. This data, from 32 adult and 11 paediatric cases is shown below. The scatter graphs are depicted with mean lines

As the GPIIb/IIIa molecule is known to exist as a dimer (Wagner et al., 1996) on the surface of the platelet it helps to confirm our reference interval as both CD41 and CD61 give similar ranges for all calculated parameters. As both mean +/- 2SD (23091 +/- 8296), median +/- 2SEM (22474 +/- 1250) and 95% confidence interval (21829-24352) are similar for CD61 and (25256+/- 8890, 25506 +/-1340, 23095-26608) for CD61 this can be accepted as a true reference range. Visually this can beeb seen in the left hand scatter plot of figure 3.13. However, the mean +/- 2SD for CD42b (7270 +/- 13230) allows for a patient with no CD42b (-5960 as bottom of range) to fall within the range. This is not correct and would not distinguish control patients to those with Bernard Soulier syndrome. The median +/- 2 SEM is comparable to the 95% confidence interval and is therefore a more realistic range. As this range is selected for CD42b, the median +/- 2SEM will be used for the other glycoproteins.

As can be seen in table 3.6, and visually in figure 3.13, the paediatric reference ranges are similar to those of the adults. The statistical power is limited due to the

small sample population. Further data needs to be tested to confirm this hypothesis.

However none of these assays are infallible. Many manufacturers use different clones to raise their monoclonal antibodies. It has been suggested that the specificity of these antibodies may affect the results obtained (Curvers et al., 2008) Results must always be considered within the limitations of each assay. The case study below illustrates this.

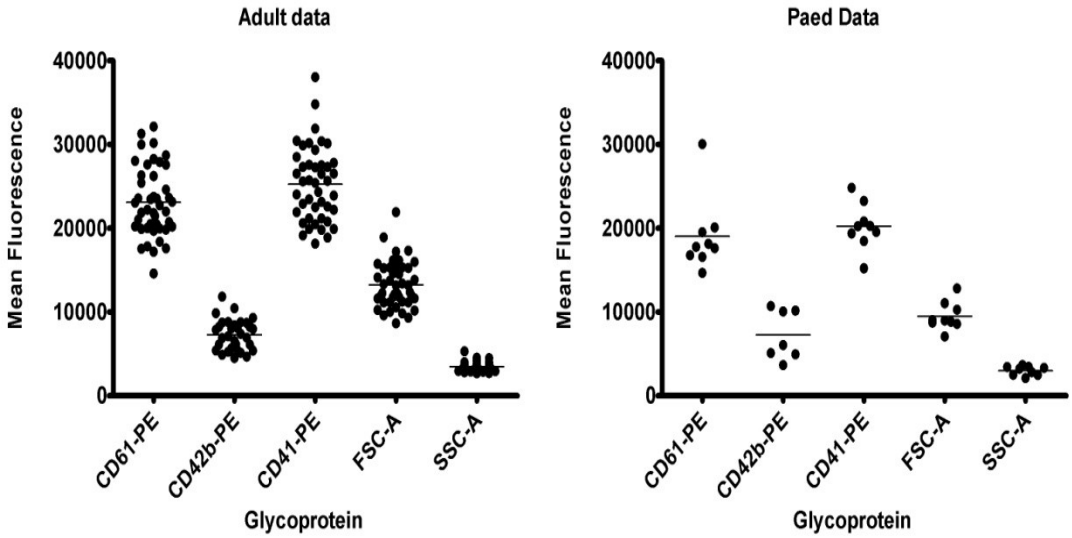

Figure 3.13: Scatter plots showing ranges for platelet glycoproteins

The plot on the left shows adult data with (from left to right) CD61, CD42b, CD41, FSC and SSC (FSC and SSC: Forward and side scatter denote the size and granularity of the platelet). The plot on the right is the paediatric data, with the same x axis

	Mean	2SD	Median	2SEM	95%CI
CD61	23091	8296	22474	1250	21829-24352
CD41	25256	8890	25506	1340	23095-26608
CD42b	7270	13230	7244	642	6615-7925

Table 3.5: Reference range for adult glycoprotein analysis

Data in the table shows the glycoprotein reference range for the adult population

	Mean	2SD	Median	2SEM	95% CI
CD61	19025	8868	17773	2956	15617-22433
CD41	20221	5474	20238	1824	18117-22325
CD42b	7240	5928	6034	2240	4498-9981

Table 3.6: Reference range for paediatric glycoprotein analysis

Data in the table shows the glycoprotein reference range for the paediatric population

Case study One:

It has been reported (Weiss et al., 1995) that the antibody (Coulter SZ21 IgG1 PE) chosen for analysis of Glanzmanns Thrombasthenia, can provide false positives. A case referred to the Bristol laboratory had been diagnosed with a 50% reduction in GPIIbIIIa in both parents and no GPIIbIIIa in the offspring. However it was established that an incorrect diagnosis of Glanzmanns Thrombasthenia had been made at the referring location. There are two variants of GPIIIa nominally called Human Platelet Antigen1 (HPA1) and HPA2 the difference being a leucine/proline switch at position thirty three. The antibody used by the referring hospital, (as well as the Bristol laboratory), only detects HPA1. This is present in 99% of the population. However it was subsequently determined that the parents of this child were phenotypically HPA1/ HPA2 and this is what gave the apparent reduction in glycoprotein numbers. The phenotype of the child was HPA2/HPA2 and therefore the antibody used in the two laboratories didn't recognise any of their GPIIIa receptors.

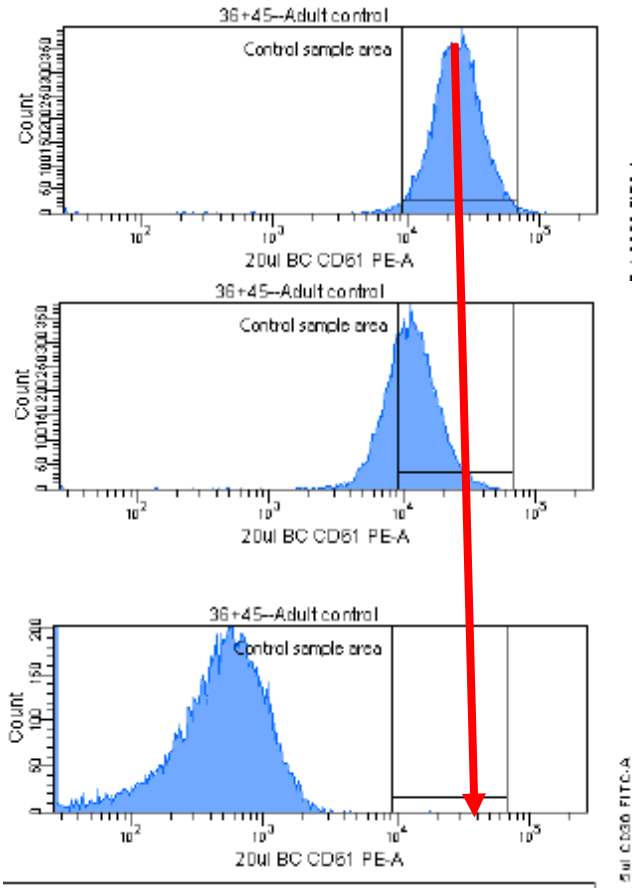

Figure 3.14: Flow cytometry data from control and family members

Top: Histogram with normal control platelets,

Middle parent 50% identification of GPIIIa

Bottom patient with no bound GPIIIa (red line shows median fluorescence peak for reference range)

Chapter 4: Discussion

4.1 Introduction

St Thomas' hospital and Bristol Royal Infirmary are both large regional comprehensive care centres for patients with bleeding disorders. At the outset of this project these centres did not have the laboratory support for dealing with platelet function disorders. To give these centres the services that were needed a review of all documented guidance was initially performed (Bolton-Maggs et al., 2006; Christie et al., 2008; Davidson et al., 1988; Harrison et al., 2011). When the project was initially undertaken, most of the guidelines were yet to be written, so reliance was placed on the British Committee for Standards in Haematology (BCSH) document of 1988 (Davidson et al., 1988). Whilst waiting for subsequent affiliated guidance to be published, autonomous laboratory research groups were publishing their own recommendations (Harrison, 2009; Hayward, 2008; Hayward & Eikelboom, 2007; Moffat et al, 2005) It should be noted that the authors on the autonomous guidance tended to be those that subsequently published similar guidance affiliated to a national or international standards body.

Several surveys from the UK (Jennings et al., 2008), Canada (Price et al., 2007) and the ISTH (Cattaneo et al., 2009) had indicated guidance was urgently needed. Also apparent from these published surveys, especially the international community survey (Cattaneo et al., 2009) was that some guidance (Bolton-Maggs et al., 2006; Christie et al., 2008; Davidson et al., 1988) was not being adhered to. This non-uniform testing led to ISTH scientific sub-committee investigation and the results of this formed Paolo Greseles' presentation in Liverpool at ISTH SSC 2012, which is discussed in section 4.2 (http://www.isth.org/default/index.cfm /ssc1/2012-ssc-subcommittee-minutes/2012-platelet-physiology-minutes)

With the rapid publishing of guidance over the last ten years, the current document may not remain the remain the de-facto standard (Harrison et al., 2011) for long. This requires a robust evidence based practice protocol. Furthermore evidence based practice is recommended as a key performance indicator (KPI) in a recent report published by the Royal College of Pathologists (RCPath)(Furness, 2011).

Implementation of the evidenced based (Baum, 2012; Mickenautsch, 2010) review methodology involved searching for primary evidence from published trials and meta-analyses that are readily available on the internet (Gurney, 2006) and a search stratagem was devised. This included an automated PubMed© (http://www.ncbi.nlm.nih.gov/pubmed) search for the phrase 'platelets' and the results delivered by email every day (See Figure 4.1 for full search details). In a personal conversation with Paul Harrison, he advised on the time limit, as large amounts of papers are constantly being generated and therefore assessing daily gives manageable results. This is augmented by an electronic table of contents search for all coagulation based journals.

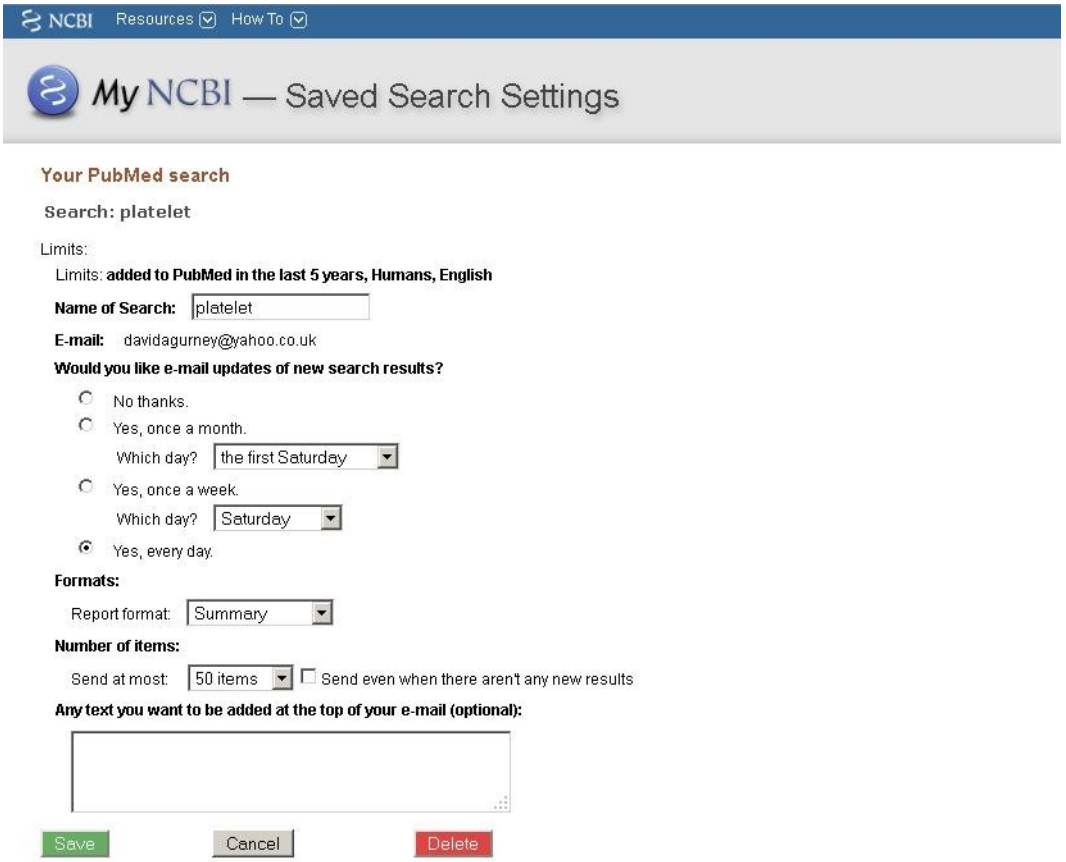

Figure 4.1 PubMed search request for literature involving platelets as part of the laboratorys' evidence based practice methodology.

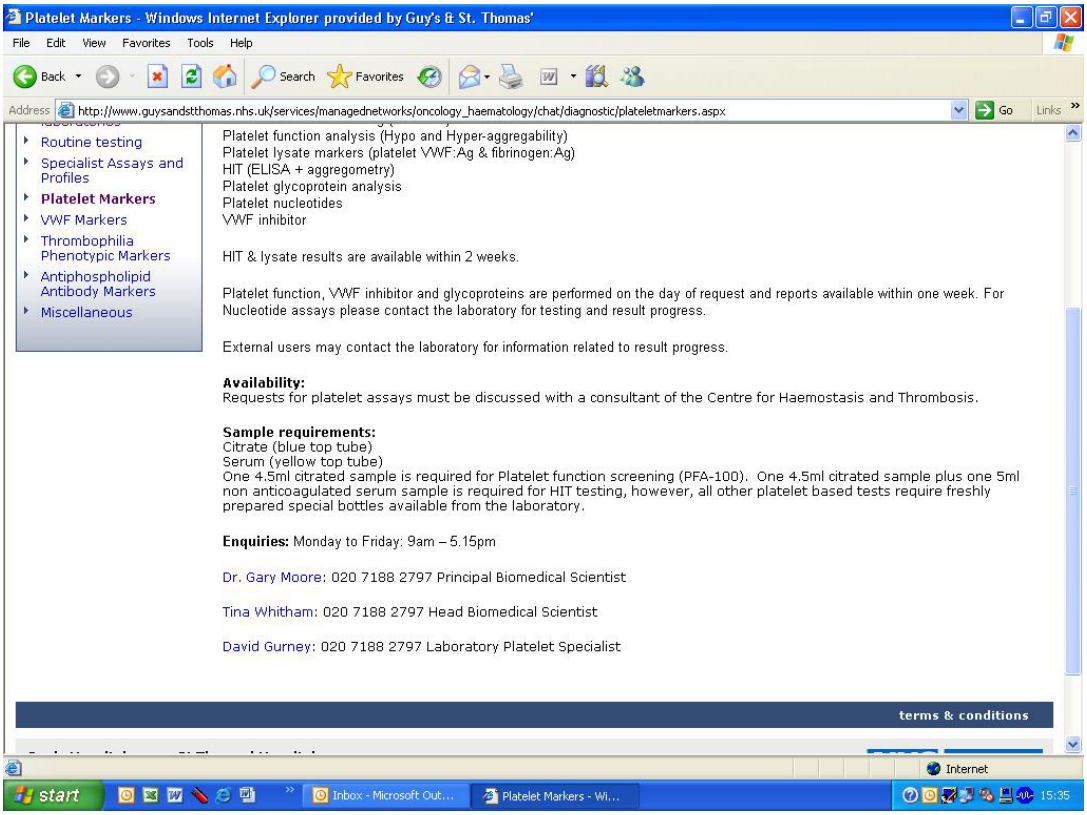

Figure 4.2: Screenshot of St Thomas Hospital Intranet page for the haemostasis laboratory showing author as point of contact for platelet work.

A single scientist responsible for the specialist platelet function testing has provided both clinical staff and scientific colleagues with a single contact point. This ensures that the process of platelet function testing (see Figure 4.3) from booking the patient to sending out the report is monitored to meet the quality standards required. Administrative staff now liaise with the nominated laboratory scientist to ensure that, as the tests involve time and technical skill, there are the staff available to analyse the samples. The nursing staff liaise with the nominated laboratory scientist to ensure the information is correct, the samples reach the laboratory without delay and the clinicians interact when requesting tests and receiving reports. This ensures continuity for the patients sample throughout the process.

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Organising Platelet FunctionTesting

1. New Patients requiring combined clinic appointment/medical review and platelet function testing

Clinician categorises as urgent or non-urgent and states whether 'standard' or 'extended' testing is required:

Non Urgent Platelet Function testing:

1) Haemophilia Centre Coordinator emails David Gurney david.gurney@uhbristol.nhs.uk if extended testing required copy in Matt Jones matthew.jones@bristol.ac.uk:
Give patient details, specify standard or extended platelet function testing, advise that a Tues am slot is needed
2) David (and Matt) agree a date (minimum of 2 weeks notice) and advise Haemophilia Centre Coordinator by email antony.woolcomb@uhbristol.nhs.uk
3) Haemophilia Centre Coordinator will provisionally book clinic appointment (1st slot), phone patient to confirm availability and send appointment with the explanatory letter and patient information leaflet to patient via standard post. (If clinic is overbooked to discuss with medic and move another patient out). Also the patient is to be entered in the Haemophilia Centre Diary for the nurses to take the blood.
4) In the week prior to appointment Haemophilia Centre Coordinator will phone patient to confirm.

Urgent Platelet Function Testing:

As above however does not need to be a Tuesday, but does need to coincide with a medic available to review.
When date confirmed by lab Haemophilia Centre coordinator will hand this over to a medic to phone the patient to explain the procedure and ensure no incompatible meds etc

2. **Known Patient having ongoing investigations for bleeding:**

The expectation is that the patient will have been seen in clinic and discussed need for platelet function testing.
After clinic medic refers case to Haemophilia Centre Coordinator who:

1) Will arrange time and date for platelet function testing (does not need to be a Tues and does not need medic review)

2) Send date and info leaflet to patient

Figure 4.3 Protocol for booking in platelet function tests at Bristol

4.2 Pre-analytical variability

Pre-analytical variability was an initial focus of this project. It has been successfully minimised by involving all staff groups in developing the questionnaire which now includes details of over the counter drugs, herbal preparations and guidance on correct phlebotomy. The results above (Section 3.2) demonstrate that the pre-analytical questionnaire has not reduced the number of platelet aggregation assays performed. The assays that are performed are able to have this information included in the report leading to more informed diagnostic decisions by the clinical team. At the 2012 ISTH meeting Paolo Gresele presented data of a survey of over two hundred laboratories, ninety two of which were clinical laboratories from thirty seven countries. In this survey 36% of laboratories gave no clinical interview, 70% performed no pre-analytical questionnaire, and 36% did not perform a normal control with each assay. There was an increased reporting of ADP defects and 34% of cases were undefined as to the cause of the patients original bleeding. The data supports the concept that 'undefined cases' could be attributed to one or more of the following: no clinical input, no pre-analytical checking or control run. Greater focus on these three items may have presented the testing laboratory with reasons for these symptoms, such as aspirin ingestion or thienopyridine prescriptions.

In light of the 2008 NEQAS report (I. Jennings et al., 2008) contact was made with distributor (Alpha Labs Eastleigh, Hants) with the suggestion that to help to rectify some of the concerns of the authors a 'user group' meeting should be convened. As the Biodata PAP-4D and PAP-8E are one of the most common platelet aggregometers available it was hoped to reach a large number of scientists active in platelet function analysis. This took place in London in October 2008

(see appendices 1-3 for agenda, presentation and report). The speakers also included Ian Jennings, author of the report (I. Jennings et al., 2008) who spoke about current practice and gave an insight into further data from the study and Paul Harrison who spoke about the then, upcoming guidelines as he was a panel member. Work on the pre-analytical questionnaire was presented and delegates present were offered it for use in their laboratories. After the meeting contact was made with eleven representatives of laboratories (see table below) and followed this up with an email 6 months later enquiring into the value and usage of the questionnaire (see appendix 4 for text).

Laboratory	Follow up
Sheffield	No reply
Abergavenny	No reply
Swansea	No reply
Ashford	Being discussed
Manchester	No reply
Bournemouth	No reply
Edinburgh	Used, locally modified
London (Royal Free)	No reply
London (Imperial)	Used in Haemophilia centre with nurses
London (UCL)	Used in SOP but not in practice
Hull	Used, locally modified

Figure 4.1: Correspondence regarding use of questionnaire

This small sample bears out the data that was being described by Gresele at the ISTH SSC 2012, detailed above. There has to be an assumption that those laboratories that did not reply to the follow up email are not using the questionnaire. It was assumed then that these places are still performing platelet function tests. This would be simple

conjecture, these laboratories may already be using questionnaires and noted there was little difference from those presented, or where at the user group to assess the suitability of setting up the assay and decided against setting up a complex assay. It is a concern however that these laboratories may be performing this test. If a pre-analytical questionnaire was to be used, rather than go through the review process prudence would dictate using an example provided? One laboratory has implemented it completely, and a further two laboratories have modified it for local use. As a standardisation tool viewing the modification would have been a useful exercise to confirm that the core questions had not been removed from the document. The final two answers are cause for concern. If the document is under discussion, but not been implemented it means that those people discussing it are unaware of guidance on the use of a pre-analytical questionnaire. If they are unaware regarding this portion of the guidance, one has to be concerned about the performance of the whole assay in that particular laboratory. Finally, a laboratory has included the document as part of their standard operating procedure (SOP) but does not use it to screen patients. This is a concern on a number of levels; firstly an SOP is a monitored document which can be audited by Clinical Pathology Accreditation (www.cpa-uk.co.uk) and if the document is included 'as is' during an audit the assessor is within their powers to ask to see examples of the completed questionnaire. Secondly the questionnaire is not being used clinically and therefore this calls into question the knowledge of the current guidelines. All the non-user laboratories are within the catchment of laboratories that are using the questionnaire and this confirms that these specialist assays should be done by specialist centres that understand the assay to the fullest extent.

Patient Questionnaire - PLATELET AGGREGATION

For first-line investigation of platelet bleeding disorders. This form must be completed by the requesting clinician and/or a member of the Haemophilia nursing team prior to platelet aggregation testing.

Surname	
Forename	
NHS Number / Hospital Number	
Date of Birth	
Telephone Number	

First Line Investigation?	
Follow Up Investigation?	
Family study? (please identify affected individual and relationship)	
Previous Platelet Aggregation Results?	

The following drugs are contraindicative to platelet aggregation testing and must be avoided for 14 days prior to date of test.
Aspirin and Ibuprofen
'Over the counter' cold relief medication
Homeopathic remedies
Prescription medication NSAIDs: Indomethicin, Sulfinpyrazone, Naproxen ADP blocker: Ticlopidine, Clopidogrel, Prasugel GPIIb/IIIa agonists: Reopro, Tirofiban, Epitifibatide, Intelligrin Prostaglandins: Cliostazol Antidepressants: Citalopram, seroxat imipramine, amitripyline Antimicrobials: Penicillins, Cephalosporins Anticoagulants: Warfarin, heparinoids, direct thrombin inhibitors
List all current medication, including any not listed above, and provide date taken
List all vitamin or dietary supplements and herbal remedies in last 10 days eg Fish oil, Ginko Biloba and Green Tea Vitamins B6, C, E

Figure 4.4a: Questionnaire as modified and used at Hull and East Yorkshire Hospital

NHS Trust (see table 4.1)

Actions to be completed by health care professional when booking platelet aggregation test	**Signature**
Identify any current medication listed above that is contraindicative for platelet aggregation – check BCSH guidelines and/or haemophilia nursing team database	
REQUESTING CLINICIAN: Advise patient to discontinue medication identified that is contraindicated	
Advise patient to avoid alcohol, caffeine and tobacco 24 hours prior to test	
Advise patient to avoid **high concentrations** of garlic, onion, ginger, ginseng, fish oil, tamarind, turmeric, vitamins C and E prior to test	
Provide patient with platelet aggregation information sheet	
Advise patient to contact Haemophilia nursing team if unwell or if contraindicative medication has been taken two weeks prior to the date of the test	
	Details of test
Date, time and location for patient to attend Details confirmed with: LAB PATIENT LOCATION	**Date** **Time** **Location**

Figure 4.4b: Questionnaire as modified and used at Hull and East Yorkshire Hospital NHS Trust (see table 4.1)

4.3 Platelet aggregation: instrument introduction and reference ranges

With regard to the instrumentation used to assess platelet aggregation in this study, the PAP-8E has a number of advantages compared with its predecessor the PAP-4D. It has eight channels, each taking 250µL of sample, whereas the earlier model had only four channels each taking 500µL of sample. This is advantageous for a number of reasons: double the number of reaction channels means that the assaying the samples takes less time, the assay is able to be performed within the recommended time-frame set in the guidelines. Doubling the channels has decreased the size of the reaction cuvettes and with this less PRP being used. This has a direct result on the amount of sample required and less sample volume being taken. The reduced sample volume is advantageous when dealing with minimal volumes required by the paediatric patients. Advances in the controlling software means that data manipulation is also easier with the newer machine. With the PAP-4D operator experience determined where the agonist was added. This led to agonists running down the side of the reaction cuvette before entering the PRP, giving a falsely elevated lag phase value. An advantage with the PAP-8E is that the sample cuvette cover is shaped to allow a pipette tip to be positioned directly above the centre of the sample, allowing the agonist to drop straight into the PRP minimising any lag phase errors.

As a new analyser and a new reagent supplier was being used, new references ranges were established for the current agonists being used in the department. The new agonists as advised by the current guidelines have been established and are now in use. On the Gaussian curve used to calculate reference ranges, there will

be 2.5% of the population at either end of the curve which, although the sampling suggests is 'normal', will be outside the reference range. For this reason, it should not be referred to as a 'normal range' but a reference range or reference interval.

The most recent platelet aggregation agonist reference data to be published (Dawood, Wilde, & Watson, 2007; Hayward et al., 2008) shows similarity to our data; however Dawood *et al* only observed the traces for a maximum of three minutes, whereas the current guideline recommends five minutes. Agonists such as epinephrine and collagen can have extended lag phases in some disease states, by not observing for a full five minutes there may be some late changes that would have been unaccounted. Hayward *et al* show a similar but tighter range for arachidonate and 5μM ADP (they don't give further dilution ranges) and for collagen, epinephrine, U46619 and ristocetin the reagent concentrations differ from those used in this study. . Other researchers found discrete populations in other agonists, also at specific dilutions.(Hayward et al., 2009)

Some variation might be expected as the data from the Birmingham group was prepared with Chronolog (LabMedics, Stockport, Cheshire) equipment and reagents, where as this data was prepared on a Biodata PAP-8E with hart Biologicals reagents. The Dawood *et al* paper only shows example traces rather than reference ranges.

The aggregometer trace was then followed for at least five minutes and the trace visually interpreted and a report written. Some of the recent guidance advocates starting with a 'screening' set of agonist dilutions (Moffat et al., 2005), and then once an anomaly is observed, to proceed to further dilutions. However as the preparation of the platelet rich plasma and the dilutions of the agonist are the time limiting stages of the assay, all dilutions of each agonist were run each time in this

study.

Another procedural discussion is on the dilution of the PRP to standardise the platelet count. Mani and Cattaneo (Cattaneo et al., 2007; Mani et al., 2005) stated that the process of adding hard spun plasma to 'normalise' the PRP was introducing factors that were interfering with the assay. This has been disputed (Castilloux et al., 2011) however the current guidance (Harrison et al., 2011) is only to dilute PRP when the platelet count in excess of 600 x 10^9/L

With low dose U46619 the final aggregation and slope are split into two distinct populations this can also be observed with final aggregation at low dose epinephrine. This low dilution is due to the agonist concentration being on the threshold of the activation limit for the platelet receptors.

In-vitro testing however, presents its own problems. Preparation of PRP removes erythrocytes and leukocytes, removing with them any part they may play in the aggregation process. It also involves centrifuging the blood sample and this activates platelets to a small degree. There are many in-vitro methods for analysing platelets, most of these are only of any worth when analysed with a control. This way you are distinguishing between how the platelets react 'in this test environment' compared to a control. This will give an indication, but no more, on how they operate within the body.

4.4 Collagen reagent comparison

The collagen comparison produced data that showed that the ABP pharmaceuticals (ABP, New Jersey, USA) collagen had a greater degree of variation in normal controls (Figure 3.9-3.11, Table 3.2) which was more pronounced at lower concentrations. This will only be of significance if the lower

limits of the range give rise to no distinguishable difference between non-responding diseased patients. Analysis of known diseased controls will be used to confirm the new agonist is able to separate the normal and diseased control populations. It should be noted that Nycomed Horm collagen is six times more expensive than the comparative product supplied by ABP pharmaceuticals (ABP, New Jersey, USA).

If after completing the diseased control study and the data shows that the broad nature of the Hart collagen reference interval means it cannot distinguish between normal and diseased patients then the laboratory will continue use the Horm product. The laboratory will inform Hart Biologicals of this finding and it is hoped that the product can be standardised to meet the Horm specifications and then the department can reduce its reagent budget. Until the diseased control study has been performed a decision to switch to the cheaper reagent cannot be made.

The fact the Horm preparation is the 'gold standard' seems to be an historical accident and reliant on the fact that twenty years ago it was the only collagen preparation available (email conversation with Alby Patterson (CEO Hart Biologicals) and Richard Farndale (Professor of Biochemistry, University of Cambridge (Farndale, 2006)).

4.5 Platelet nucleotides reference ranges

Platelet nucleotide analysis is a useful adjunct to nucleotide release analysis as performed on the Chronolog analyser (LabMedics, Stockport, Cheshire). The Chronolog analyser measures luminescence as produced in a PRP sample after activation with an agonist, most commonly TRAP, this is a representation of the

granule release mechanism. If the granule release mechanism is defective, then the Chronolog analyser will product results indicative of delta storage pool disorder. Upon measuring platelet nucleotide content directly as described above in section 2.4, a normal result will be obtained. These results coupled together give the information that there is an enzymatic cascade problem in the release machinery, such defective PKC isomers.

With the an expert clinical team and technical proficient laboratory service, the Bristol Haemophilia centre has been selected as a quaternary referral centre for Hermansky Pudlak disorder and is recommended by Hermansky-Pudlak Syndrome Network UK (http://www.hpsnetwork.co.uk/) a support network for HPS patients.

The nucleotide assay is a quantitative test; therefore the median +/- 2SEM is a sensible way to produce a reference interval; however it would not be useful to construct a range for the platelet aggregation in the same way. The small population size and the qualitative nature of the assay mean that the mean +/- 2SD is the correct way to calculate this.

4.6 Flow cytometric glycoprotein reference ranges

Flow cytometry for platelet glycoproteins is now offered as a routine test to all the specialist haematology clinical teams. In this study patients who had normal aggregation results were included in the reference population for the glycoprotein reference interval. This practice increases the number of subjects in the reference pool more quickly than just using control subjects. However one has to consider that these patients have had other platelet function tests performed as their

symptoms dictate a primary bleeding disorder. So, if the patient has a positive bleeding history, should they be used as a control? The data in the results section (Table 3.5) indicates that this was an acceptable practice as the reference interval is quite narrow with few outliers. If this data had presented with a large spread or a number of outlying results then this approach would have been re-considered. Published data on these reference ranges (Ozsavci et al., 2002) state that GPIIb was 43828+/- 7904, GPIIIa 42351+/- 1049 and GPIb 22166+/- 3847. The data was constructed using an antibody conjugated to fluorescein isothiocyanate (FITC) fluorochrome. This fluorochrome does not have a one-to-one ratio with its epitope and therefore the count is unreliable, this paper based the reference range on five normal cases, this is not statistically satisfactory.

Further analysis is needed to establish the reference interval for the paediatric controls; the current group does not have sufficient power to create a range. There are however, obstacles in obtaining paediatric control samples; these include ethical issues of collecting blood from healthy paediatric patients and population size. Population size is directly related to the ethical issue.

4.7 Integration into a specialist laboratory environment

In 2000 during a conversation with the Clinical Director of the haemophilia centre at St Thomas' Hospital it was mentioned that only 40% of patients who have a laboratory demonstrable platelet function disorder receive a clinical diagnosis. In the work presented here, there has been development of sufficient expertise to increase that value. Platelet aggregation is now listed in the department's first line screening protocol and is available as part of the laboratory's routine repertoire.

Previous to 2010 in Bristol, platelet aggregation was considered a second or third line specialist screening assay. This assay is now available to a greater number of patients, allowing diagnoses to be made in a shorter timeframe, meaning fewer hospital attendances for the patient. This saves time for the patient, and time and resources for the hospital.

In a recent study (Hayward et al., 2012) it was reported that adding LTA and VWD screening to first line bleeding disorder investigations increased the sensitivity from 3.7% to 30% (whilst maintaining specificity i.e. slightly reduced 96% to 88%), compared to using just a coagulation screen which included a prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen (by the Clauss method). There study also found that 36% of the definite bleeding disorders had no demonstrable cause, showing that there is still work to be done twelve years after the conversation with the St Thomas' Clinical director.

Integration of the service can be demonstrated by the fact that there was a single point of contact for platelet work at St Thomas' hospital (Figure 4.2). This model has been repeated for the integration of service at Bristol Royal Infirmary (See Figure 4.3). Pre-analytical variability has been reduced by collaborating with all staff groups, a process that was driven by and centred on the laboratory (Section 4.2).

New aggregation equipment and new reagents have been introduced, standardised and reference ranges generated as driven by evidence based practice (Section 4.3). Studies have been undertaken to decrease the cost of the assays in the laboratory. Dependant on the results of further testing the cost of the collagen reagent could decrease significantly (Section 4.4).

The flow cytometric analysis of glycoproteins has been brought ‘in-house’, improved and standardised and is now offered as a routine assay to specialist haematology clinical staff. This ensures the turnaround time has reduced from a week, to 8 hours.

Point of care testing has not been integrated into the department; however rotational elastometric (ROTEM) assays are used in theatres for rapid assessment of the patient undergoing surgery but has not been added to the laboratory diagnostic repertoire.

All of these components mean the laboratory can fulfil every stage of the algorithm (Figure 1.4) presented by UK Haemophilia Centre Directors Organisation (UKHCDO) paper (Bolton-Maggs et al., 2006) for diagnosing platelet disorders meet all the recommendations for diagnosing platelet function disorders in the latest guidelines (Harrison et al., 2011). The department has a dedicated molecular genetics unit on site for genetic confirmatory diagnosis of diseases covered in section 1.2.3. Should the need arise expertise in the fields of electron microscopy, western blotting and plasmid manipulation are available through collaboration with the Bristol Platelet Group, a specialist research team at Bristol University (www.bristolplatelets.org). Genetic analysis is more frequently performed as confirmation of a phenotypic analysis such as platelet aggregation or platelet release. In the current economic climate, electron microscopy tends to be used for absolute confirmation of new or rare disorders. This interaction between the diagnostic hospital laboratory and the university research laboratory is aiding the development of translational medicine. That is techniques that were the preserve of research laboratories, being used in specialist hospital laboratories for advanced diagnostic purposes. Polymerase chain reaction (PCR) genetic analysis

is a recent example of this.(Jones et al., 2011)

Coupled with all the above, working with an expert team of clinicians and other scientists, there is now co-ordination of an advanced laboratory service for the benefit of haemophilia centre patients.

4.8 Recognition as a specialist service

The laboratory has been recognised as expert by a number of the external organisations. Biodata approached the laboratory when it was preparing its user group (see appendix 1-3 for agenda). Hart Biologicals approached the laboratory for advice on platelet function kits and its collagen reagent (section 4.4). The Hermansky Pudlak network (http://www.hpsnetwork.co.uk/) a patient support group approached the clinical team to operate as a tertiary referral centre for their members to confirm previous diagnosis. The improved laboratory has enabled it to participate in national studies, such as genotyping and platelet phenotyping (GAPP) study (http://public.ukcrn.org.uk/search/StudyDetail.aspx?StudyID= 9858). This study is using phenotypic diagnosis to direct gene sequencing and analysis so that a genetic diagnostic portfolio can be developed. The GAPP study is a UK collaborative effort between the laboratories in Bristol, Birmingham and Sheffield, and international studies, such as the Bridge (https://bridgestudy.medschl.cam.ac.uk/whatis.shtml) study. This an international collaboration between UK, European and US laboratories building on the work the GAPP study researching genetic causes of rare platelet disorders. The collaboration has already yielded results in locating the causative gene in gray platelet syndrome (Albers et al., 2011).

A number of the services' staff are now representatives on national (David Gurney, IBMS Haematology Specialist Advisory Panel) and international (Andrew Mumford, Platelet physiology ISTH SSC) committees. In my role as specialist scientist at Bristol and specialist advisor for the IBMS, invitations to speak have been offered at IBMS Biennial congresses, and accepted in 2005 (appendix 7), 2009 (appendix 8) and 2011 (appendix 9). These presentations have led to an invitation of collaboration for a book chapter (Moore & Gurney, 2010) on laboratory diagnosis of platelet function disorders.

In his review of pathology service Lord Carter of Coles (Carter, 2006) recommended that specialist services be consolidated in areas of expertise. The laboratory at Bristol is now well placed to be one of those specialist laboratories. It has a comprehensive care centre for bleeding disorders on site, staffed by expert clinicians (A. D. Mumford, 2009) and a scientifically accomplished laboratory service.

In the future we will continue to offer the best evidence based analysis for platelet function disorders. Phenotypic testing is likely to reduce over this period as more is discovered about platelet genetics. Just as genetic testing will increase, so will our knowledge of platelet structure (Zufferey et al, 2011) Bristol Royal Infirmary is well place to build on this with a dedicated genetic laboratory (Jones et al., 2011). Furthermore assays for deficiencies in particular proteins (or even groups of proteins) will become common place. All the proteomic data (Di Michele, Van Geet, & Freson, 2012) and genetic information (Freson, Izzi, & Van Geet, 2012) will yield numerical and structural results which may infer functional abnormality. Platelet aggregation is still the gold standard method that shows how they

function. Borns' (Born, 1962) invention is still relevant sixty years on. Figure 4.5 shows the departments 'expert centre' entry on Ophanet, a database of the rare diseases network collated by INSERM under the auspices' of the European Union.

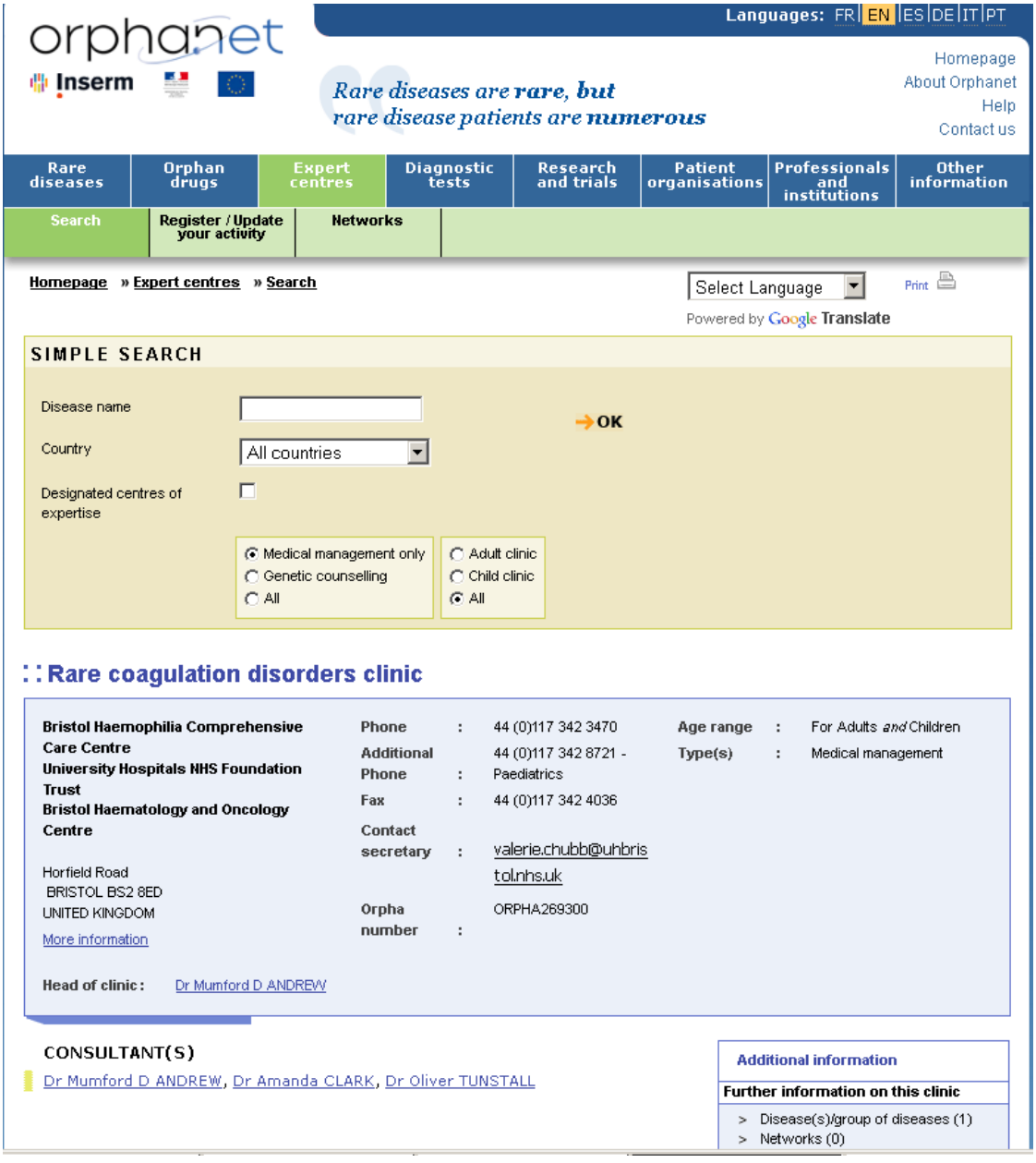

Figure 4.5: Bristol Royal Infirmary listing as an expert centre entry on Orphanet.

Chapter 5: Reflection and Dissemination

5.1 Reflection

The professional doctorate as offered by the University of Portsmouth is a two component course; the first part comprising of a set of taught modules with the final part consisting of the project. The taught modules are broadly divided into four units:

- Professional review and development. This is a critical reflection on what has been achieved to date with a learning contract to build in what is needed for future learning
- Advanced research techniques. This involved learning a broad range of quantitive and qualitive research techniques and included an intermediate statistical module.
- Publication and dissemination. This involved a critical appraisal of previously published journal articles, preparing articles for submission and reviewing other candidates' submissions in the role of journal editor.
- Project proposal. To produce a research proposal for this thesis (included as appendix 11)

There are a number of learning theories and associated styles. The first one encountered and the one that seemed particularly relevant whilst undertaking the course was Kolbs' theory (Kolb, 1984) This model of four points of learning and the relevance to my learning experience;

- *Unconscious incompetence*. In this instance being unaware of the existence of the DBMS course

- *Conscious incompetence*: Being aware of the course at University of Portsmouth but unsure of the requisite requirements to start studying.

- *Conscious competence*: Moving from conscious incompetence to conscious competence is the move that most people would equate to 'learning', the act of doing something to change the perception of a given topic. This equates to actively searching out and researching a topic.

- *Unconscious competence*: The ability to informally learn, that is acquiring information on a topic from outside an official learning environment.

This was summed up succinctly by Donald Rumsfeld in 2004, with his speech, 'we know what we know. We know what we don't know. We don't know what we don't know'.

Informal learning is fun; the process of formalisation of informal learning is an anathema. Reflective practice is something that any good scientist does on a regular basis, analyses what is known and unknown and what the incident has taught and what has now become a known value. The movement towards providing evidence that this task has been done, providing written reports on what has been learned, seems to strangle the joy of learning from the process. This formalisation of learning could make the career path unattractive for some students.

The professional review module and the publication and dissemination module were of particular interest. Working with a mentor who introduced me to the publication process and with an interest in IT, the open access debate is something that was followed closely. When it came to writing about impact factors and open access the submitted course work benefitted from an informal interest and was

reflected in the marking.

Teaching forms a large part of an advanced biomedical scientists role, so the knowledge that different people teach and learn in distinctly different ways has helped to orientate the appropriate teaching and tutoring methods to the learning needs of the individual under instruction.

Instruction at different levels requires different skills. Preparing a clinical colleague as a candidate for MRCPath examinations requires a different skill set and level of teaching compared to teaching medical laboratory assistants. Clinical colleagues require science based tutoring where as laboratory assistants need to be made aware of reagent detail, quality assurance and sample requirements. Inclusion of demonstration of applications and then letting students try that for themselves. Being aware that other people need to make notes on a task or need to watch a task being performed many times to absorb the information. If this helps the student to learn then this is the way it will be demonstrated.

Figure 5.1 below shows my learning style which is verbal, visual and logical with no physical or aural components.

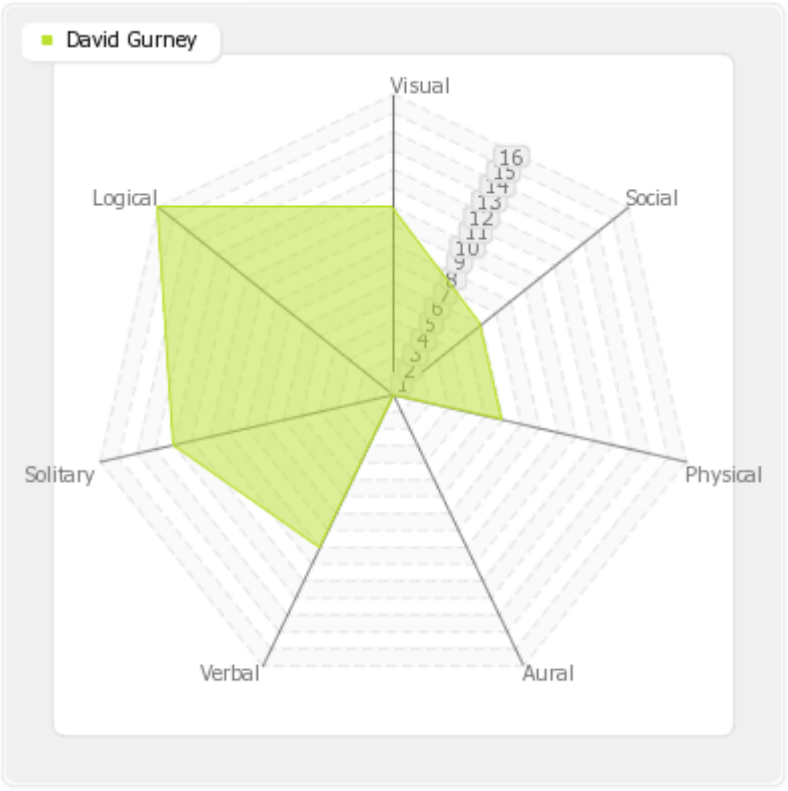

Figure 5.1: Learning graph for David Gurney

A PowerPoint exercise (See appendix 4) that enabled trainees to revise outside the laboratory environment, reaffirming the theoretical concepts after a practical demonstration. Removing an experienced scientist from a laboratory leaves a large gap of knowledge. An in-house training presentation (appendix 6) was given and a platelet interpretation manual (see appendix 10) was written in order to ease the transition of the staff taking on the role that had been vacated.

Presentation skills can only be learned by actually doing them. Previously I have only presented once at the UK Platelet group meeting in Leicester in 1999, the course honed these skills by requiring regular presentations to the academic group as part of the learning experience. These presentations in a safe friendly environment increased confidence in the ability to perform these tasks. That

increase in confidence has resulted in invitations to present at Institute of Biomedical Sciences (IBMS) biennial congress detailing both platelet diagnostic work (case presentation 2005, 2009; appendices 7 and 8) and platelet development work (platelet analysis; 2011; appendix 9). The data in these presentations is directly related to the work carried out in this thesis.

In gaining the confidence to present to the wider profession the data presented was advising the profession on laboratory policy. This transition to policy advice meant that when offered a seat on the IBMS Haematology specialist advisory panel, this was accepted. Subsequently the chair of the IBMS advisory panel was offered and accepted. Special advisory panels are in place to advise both the IBMS executive committee and the membership on a range of topics such as quality, training, registration, safety and emerging technologies. It has the remit to organise and advise on conference content, Institute academic examinations and nominate individual for both internal and external committees.

Learning new techniques, such as flow cytometric analysis of platelet markers, platelet nucleotide analysis and nucleotide release analysis, has provided a better understanding of both the diagnostic process and the functioning of the platelet.

The construction of the questionnaire has meant having to communicate with many levels of staff (detailed in 3.1).For example this has involved explaining to phlebotomy staff why the samples for this assay have to be taken in a specific way and explaining to nurses the need for thorough completion of the questionnaire. I was also involved with consultant staff in the initial review of the questionnaire and its use.

In this age of consolidation (Carter, 2006) the specialist laboratory will be subject to rigorous scrutiny. The expert scientists must deliver value for money, by

ensuring that only the correct tests are done, eliminating wasteful testing to reduce time and resources. Additionally co-ordinating these tests so that efficient use of samples, so as to minimise inconvenience to both patient and clinical team. It must be cost effective, but deliver results in a timely manner. In conclusion, the laboratory at Bristol is now able to deliver by a specialist clinical and scientific team all on one site.

5.2 Dissemination

Dissemination of knowledge has been demonstrated previously in section 4.7, however to summarise:

Training has been given at local levels in both Bristol. (Appendix 5) and St Thomas' (Appendix 6 and 10) across a broad range of staff groups

Presentations have been given at local level (Appendix 6) and national events (Appendix 7-9)

Interaction with both national (IBMS Haematology advisory panel) and international (ISTH Platelet physiology SSC) policy making

Published work in text book (Moore & Gurney, 2010)

(http://www.amazon.co.uk/Haematology-Fundamentals-Biomedical-Science-Moore/dp/0199568839) a book which has been purchased by the department for teaching and consequently by a number of the students.

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Preview Sample Pages
 This document provides concrete, standard procedures for using aggregometry to assess platelet function in patient specimens with the intent to achieve

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Appendices

Appendix 1: Agenda for PAP User group

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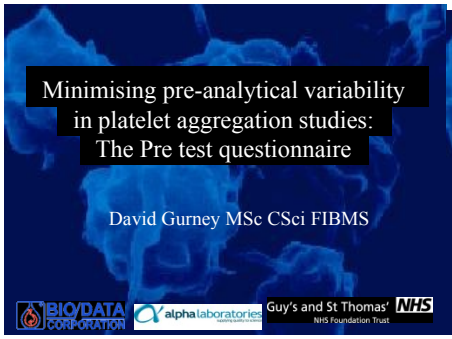
PAP USER GROUP

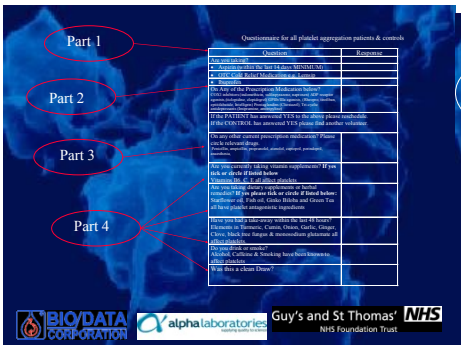
AGENDA

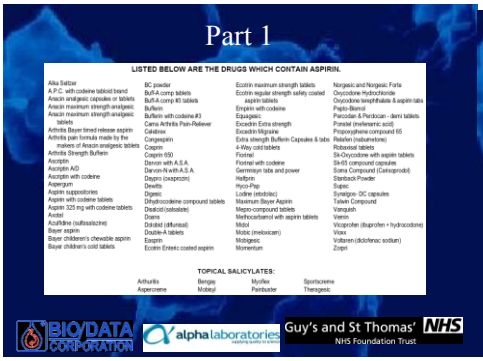
PAP User Group Meeting
Venue: Institute of Chartered Quality, London
Date: Tuesday 28 October 2008

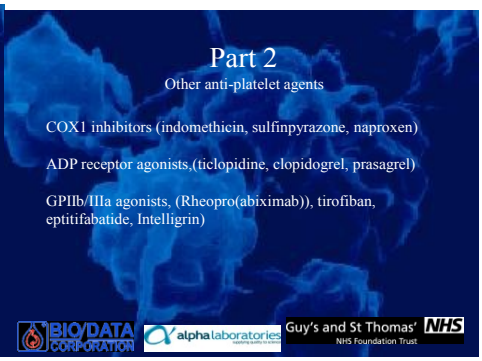
Time	Subject	Speaker
10.00 onwards - Coffee and Registration		
Session 1: Pre-Analytical Factors Chair - Chris Hughes St James University Hospital Leeds (provisional)		
10.30	Platelet Formation	Paul Harrison Churchill Hospital
11.00	Pre-Aggregation Questionnaire (Substances that can affect platelet aggregation)	David Gurney St Thomas' Hospital
11.30	PDQ An evaluation	Kampta Sukhu Churchill Hospital
11.50	Standardisation of Pre-Analytical and Analytical Variables in Platelet Function Testing	Kevin Horner Royal Hallamshire Hospital
12.15	Q & A	All
12.30 Lunch & Networking		
Session 2: Testing Chair - Andrew Chitolie, University College London		
13.45	Snake Venom & Aggregation	Gary Moore St Thomas' Hospital
14.15	Platelet Function Testing -Current practice in the UK	Ian Jennings UK NEQAS (Blood Coagulation)
14.45	The US experience CAP Survey	Bill Trolio Bio/Data Corporation
15.15	Guidelines/Standardisation Past, Present & the Future	Paul Harrison Churchill Hospital
15.45 Afternoon Tea		
16.00	Questions & Answers PAP Maintenance & Troubleshooting	Staff from Bio/Data and Alpha Laboratories
16.30 Close		

Appendix 2: Presentation for PAP User group









Part 3

Other prescription drugs

On any other current prescription medication?

Penicillin:	Reversible prevention of GPIb/VWF binding
Ampicillin	*Beta Lactam Antibiotics* Blocking surface receptors
Propranolol/Atenolol	*Beta blockers* Inhibition of secondary aggregation and release

Part 3

Other prescription drugs

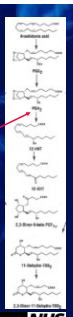
Captopril/Perindopril	*ACE Inhibitors* Decrease in TxA2
Anaesthesia	Halothane
Imipramine/ Amitripyline	*Tri-cyclic antidepressants* Inhibit aggregation responses to ADP, epinephrine and collagen
Prozac	

Part 4

Other agents affecting platelet function: Vitamins

Vitamin B6 down regulates GPIIb/IIIa

Vitamin E interferes with Arachidonate metabolism

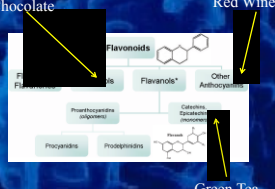


Part 4

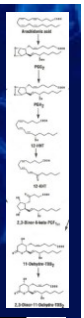
Other agents affecting platelet function: Diet

Chocolate

Red Wine



Green Tea



Part 4

Other agents affecting platelet function: Diet

"Mediterranean diet"

Red Wine

The "Wine, Nutrition & Health" Unit of the "Office International de la Vigne et du Vin", intergovernmental organization, Review. **Alcohol, wine and platelet function.** Biol Res. 2004;37(2):209-15

Onion/Garlic inhibit collagen binding and cyclo-oxgenase activity

M. Ali, T. Bordia, T. Mustafa Effect of raw versus boiled aqueous extract of garlic and onion on platelet functions. ***Prostaglandins, Leukotrienes and Essential Fatty Acids***, *Volume 60, Issue 1, January 1999, Pages 43-47*

Part 4

Other agents affecting platelet function: Diet

Ginger: Reduction in thromboxane production

M. Thomson et al. The use of ginger (Zingiber officinale Rosc.) as a potential anti-inflammatory and antithrombotic agent Prostaglandins, Leukotrienes and Essential Fatty Acids, Volume 67, Issue 6, December 2002, Pages 475-478

Clove: Competition for arachidonate

K. C. Srivastava Antiplatelet principles from a food spice clove (Syzgium aromaticum L) Prostaglandins, Leukotrienes and Essential Fatty Acids, Volume 48, Issue 5, May 1993, Pages 363-372

Turmeric/Cumin: Disruption to thromboxane biosynthesis

Shah et al. Inhibitory effect of curcumin, a food spice from turmeric, on platelet-activating factor- and arachidonic acid-mediated platelet aggregation through inhibition of thromboxane formation and Ca2+ signaling. Biochem Pharmacol. 1999 Oct 1;58(7):1167-72

Part 4

Other agents affecting platelet function: Diet

Starflower oil: High in Omega 6 and Soy Lecithin

Green Tea: catechins inhibit collagen arachidonate & U46619

Dong-Ju Son et al. Antiplatelet effect of green tea catechins: a possible mechanism through arachidonic acid pathway. Prostaglandins, Leukotrienes and Essential Fatty Acids Volume 71, Issue 1, July 2004, Pages 25-31

Part 4

Other agents affecting platelet function: Diet

Daily prickly pear consumption improves platelet function

R. Wolfframa, A. Budinskyb, Y. Efthimiouc, J. Stomatopoulosc, A. Oguoghod, I. and H. Sinzinger Prostaglandins, Leukotrienes and Essential Fatty Acids Volume 69, Issue 1, July 2003, Pages 61-66

Platelet activating factor and lyso-phosphatidylcholines from strawberry.

Calligerou M, Siafaka-Kapadai A, Galanopoulou D, Weintraub ST, Mavri-Vavayanni M. Phytochemistry. 1996 Jan;41(1):89-92.

Acai

Part 4

Other agents affecting platelet function: Lifestyle

Smoking

A. D. Blann, et al. (2001). 'Increased platelet glycoprotein V levels in patients with coronary and peripheral atherosclerosis-the influence of aspirin and cigarette smoking.'. *Thrombosis and haemostasis* **86**(3):777-783.

Alcohol,

C. R. Pace-Asciak, et al. (1996). 'Wines and grape juices as modulators of platelet aggregation in healthy human subjects'. *Clinica Chimica Acta* **246**(1-2):163-182.

Exercise

El-Sayed, Mahmoud S 1; Ali, Nagia 2; El-Sayed Ali, Zeinab (2005) **Aggregation and Activation of Blood Platelets in Exercise and Training.** Sports Medicine. 35(1):11-22..

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Part 4

Other agents affecting platelet function

Sampling

- Large gauge needle
- Discard first 1mL
- Use syringes

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Overview

Patient answers yes to part 1 → reschedule

Patient answers yes to part 2 → assess with clinician

Patient answers yes to parts 3 and 4 → check for multiple interactions

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AN AGGREGATION OF PAP USERS

PAP Analysers - The main attraction

The first user-group meeting report

It all began as a question...

Whilst attending ISTH Geneva and chatting with Carl (Sorace) I would ask a question and he would say 'Oh you know so and so, they use it?' I would continually reply that I didn't know these people and Carl promised to introduce me.

Whilst having a discussion later in the bar that involved mistaking litres for pints and drinking too much German lager, a plan was hatched to get all these people I should meet into one room. 'Why don't we have a user group?' I foolishly said 'An excellent idea', my drinking companion responded; let me ask at the company for the OK. Carl obtained not only 'the OK' but enthusiastic support for the idea from Alpha and the organisational machinery was put in place. Dates, venues and speakers were proposed, discussed and booked.

High Quality Meeting

So on a bright cold morning in October thirty five delegates descended on the beautiful regency headquarters of the Chartered Quality Institute for the inaugural PAP users' group meeting.

Matthew Davis (Alpha Labs) welcomed everyone to the meeting and handed over to the chair for the first session Chris Hughes (St James Leeds). He proceeded to take charge of a fascinating session entitled 'Pre-analytical Factors' Opening with Paul Harrison (Churchill Hospital, Oxford) describing the work being done on platelet formation and the presentation of some spectacular video footage of platelet development. I followed (David Gurney, St Thomas Hospital, London) describing the pre-analytical questionnaire that we developed at St Thomas to aid diagnosis.

Kampta Sukhu (Churchill Hospital, Oxford) followed with data from their trial of the PDQ for use when

Trial of the PDQ by Churchill Hospital Oxford

performing aggregation studies. The final presentation of the morning was given by Kevin Horner (Royal Hallamshire, Sheffield) on standardisation of pre- and peri-analytical variability in aggregation studies.

The session finished with a lively Q&A session. An excellent lunch was provided by the venue and opportunities to discuss the morning's session and network were available. I spent a lot of time discussing my questionnaire with interested parties!

Biting comments

For the afternoon session entitled 'Testing', Andrew Chitolie (UCL London) chaired and introduced my colleague Gary Moore (St Thomas Hospital, London) as the first speaker. Gary gave a talk on snake venom and platelet aggregation, using many pictures of the snakes themselves and the effects of their bites! Ian Jennings (NEQAS Sheffield) spoke next and confessed to being somewhat nervous as he was ophidiophobic. Ian shouldn't have worried; he gave an excellent talk on the NEQAS survey regarding platelet aggregation and how little standardisation there seemed to be between laboratories.

Bill Trolio from BioData gave a talk about the US standards system and the manufacturers place within that. Finally, Paul Harrison (Churchill Hospital, Oxford) took to the stage once again to discuss the various platelet testing guidelines that are in process of being written. This was all thirsty work and tea was welcome. After tea, a Q&A session with Alpha and BioData representatives produced some interesting questions.

I believe the day was a success and want to thank Carl, Chidi and Matthew at Alpha for all their input and hard work. Thanks also to the speakers and the delegates for making it so interesting and rewarding. I know many more people now that are using the PAP analysers!

Article by David Gurney (Haemophilia Reference Centre - St Thomas Hospital)

For more information on PAP8-E & PDQ - Click here for the online reply card

Back to email content

Appendix 4 : Questionnaire follow up email

From: Gurney David <David.Gurney@gsts.com>
To: Ian Ford <Ian.Ford@uhl.nhs.uk>; "Manning, Richard A" <RichardA.Manning@imperial.nhs.uk>; Ann Fisher <Ann.Fisher@UHSM.NHS.UK>; Mark Thomas <Mark.Thomas@asph.nhs.uk>; "Ali, Iftikhar" <Iftikhar.Ali@btuh.nhs.uk>; Nick Dorward (ABMU NHS Trust - Haematology) <Nick.Dorward@wales.nhs.uk>; "Scott, Richard" <Richard.Scott@rbch.nhs.uk>; "Smith, Margaret (NHH)" <Margaret.Smith2@gwent.wales.nhs.uk>; "Brooks, Sarah" <Brooks@royalfree.nhs.uk>; catriona crow <catriona_crow@yahoo.com>
Sent: Tuesday, 12 May, 2009 14:32:09
Subject: Platelet Questionnaire

Hi all

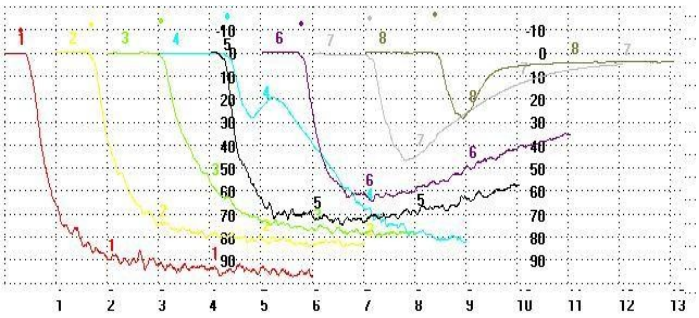
At the PAP User group meeting last October you all expressed a preference in using my pre-analytical questionnaire? I am now looking at writing up my thesis and would be grateful if you would answer some questions?

- Did you find the questionnaire useful?
- Do you use it routinely or as a guide?
- Has it become part of your SOP?

Any further info would be much appreciated:-))

Cheers

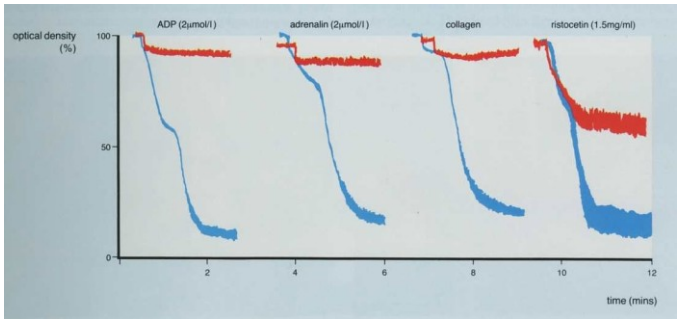
Appendix 5: Questions for MRCPath candiadtes



This trace shows traces with various concentrations of ADP, progressing from high to low. Traces 1-4 are the control patient. Traces 5-8 are the patient.

What could the diagnosis be?

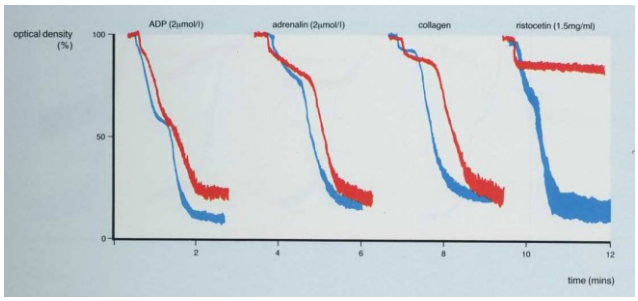
What further tests would you do to confirm your diagnosis?



This is a trace from a different model of aggregometer. The patients traces are in red and the control in blue. The agonists are marked above the trace lines.

What is the possible diagnosis?

What further tests would you do to confirm your diagnosis?

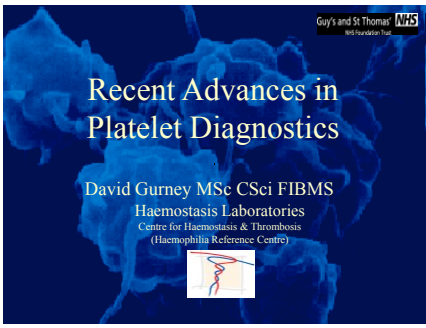


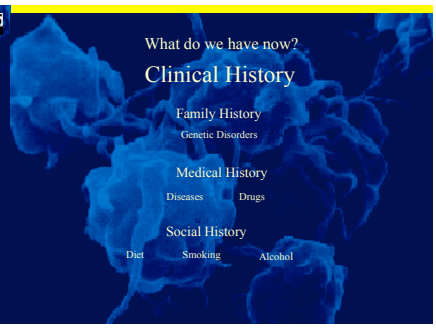
This is a trace from the same model of aggregometer as above. The patients traces are in red and the control in blue. The agonists are marked above the trace lines.

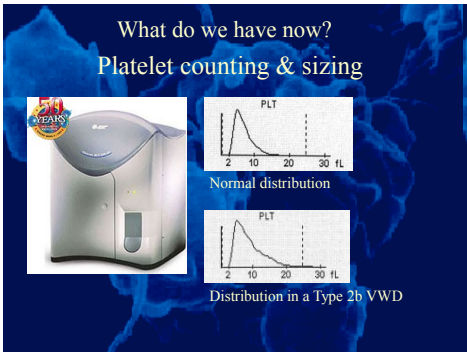
What is the possible diagnosis?

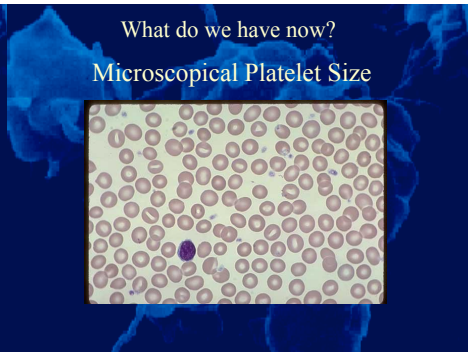
What further tests would you do to confirm your diagnosis?

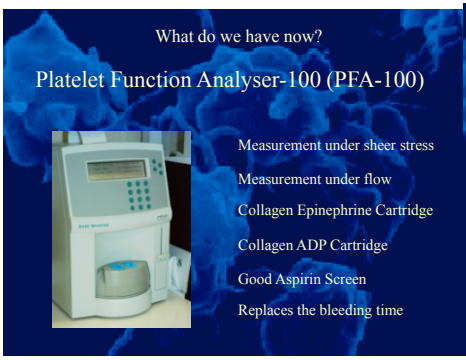
Appendix 6: ‘Recent advances in platelet diagnoses’ presented at St Thomas’ hospital

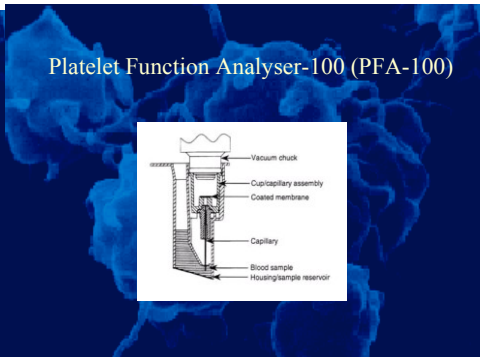




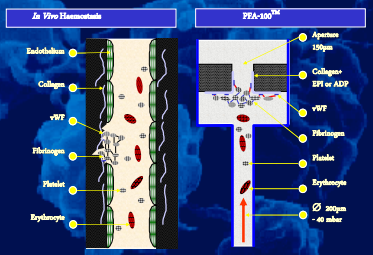








Vasculature vs PFA.



What do we have now?

Platelet Aggregation Profiler

Born GVRF Aggregation of blood platelets by ADP and its reversal

Nature 1962; 194: 927 - 929 1

Agonists

What do we have now?

- Arachidonate Epinepherine
- Ristocetin ADP
- Collagen

How can we improve on 'now'?

- Thrombin Receptor Agonist Peptide (TRAP)
- U44619 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α
- A23187 Calcium Ionophore
- Convulxin specific GPVI agonist
- Collagen receptor peptides (CRP)

What do we have now?

Nucleotide Analysis

Nucleotide Analysis

Activation of Luciferin

ADP + Phospho-enol Pyruvate (PEP) ----> ATP Pyruvate Kinase

Luciferase + Magnesium Ions

ATP + Luciferin ----> adenyl Luciferin + LIGHT

Flow Cytometry

Glycoprotein Analysis

- **Platelet CD Reference Chart**

CD	GP	Integin	Supplier	Direct
CD29	GPIIa	Beta1	Serotec Cat No MCA1949F	Y
CD31	GPIIa/PECAM		Serotec Cat No MCA1738F	Y
CD36	GPIIIa/IV		Serotec Cat No MCA722F	Y
CD41	GPIIb	alpha2b	Serotec Cat No MCA467F	Y
CD42a	GPIX		Serotec Cat No MCA1227F	Y
CD42b	GPIb alpha		Serotec Cat No MCA740F	Y
CD42c	GPIb beta			
CD42d	GPV			
CD49b	GPIa		Serotec Cat No MCA743F	Y
CD49e	GPIc	alpha5	Serotec Cat No MCA698F	Y
CD49f	GPIc		Serotec Cat No MCA1457F	Y
CD61	GPIIIa	Beta3	Serotec Cat No MCA728F	Y
CD62P	P-Selectin		Serotec Cat No MCA796F	Y
PAC-1			BD Bioscience Cat No 340507 (IgM)	N
PAR1			Zymed Cat No 35-2200	
GPVI			Gift from Moroi	N

How can we improve on 'now'?

Diamed Impact-R

Platelet function screen

Less volume than PFA-100

Adjustable sheer rates

Technically more demanding than PFA-100

How can we improve on 'now'?

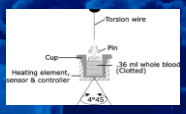
Chronolog 700

- Less manipulation
- Lower blood volume
- Involvement of other cellular components
- Release studies & aggregation

Technically demanding

'New for old'?

Haemoscope TEG

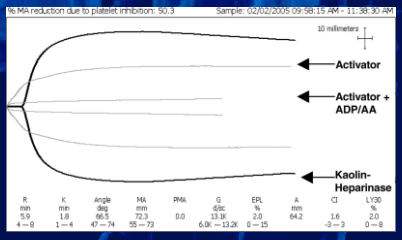


Global haemostatic overview

Clot properties only

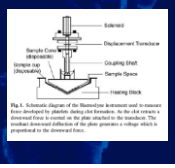
Insensitive to aspirin

Haemoscope TEG



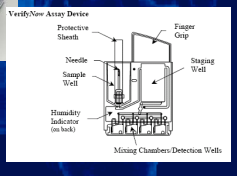
Next Generation?

Haemodyne 'Platelet Analysis System'

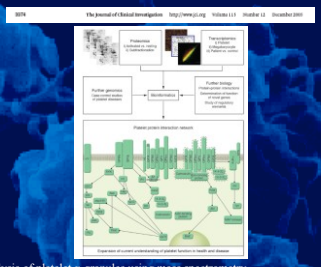


Next Generation?

Point of Care Platelet Testing



The future: proteomics and genomics?



Proteomic analysis of platelet α-granules using mass spectrometry
Authors: MAYNARD, D. M.1; HEIJNEN, H. F. G.2; HORNE, M. K.3; WHITE, J. G.4; GAHL, W. A.1.
Source: Journal of Thrombosis and Haemostasis, Volume 5, Number 9, September 2007 , pp. 1945-1955(11)

Expanding our test repertoire

- Scotts Syndrome
  - » Membrane disorder
- Quebec platelet disorder-
  - » raised uPA
  - » Reduced platelet FV
  - » Reduced multimerin
- May Hegglin Anomoly
  - » MYH9
- Sebastian Syndrome
  - » Increase in GPIIb/IIIa binding
  - » Decrease in GP Ib/V/IX binding

Appendix 7: 'Case Presentation' presented at IBMS congress 2005

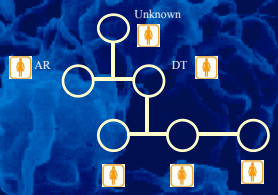
Case Presentation

David Gurney MSc CSci FIBMS

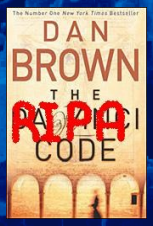
Haemostasis Laboratories
Centre for Haemostasis & Thrombosis

Guy's and St Thomas' NHS
NHS Foundation Trust

Family Tree



A Tale of a Maternal Bloodline



AR

- Female, born 1967
- Pregnant 1999
- Noted platelet count of $< 50 \times 10^9$/L
- Assumed to be ITP
- Treated with steroid, but no improvement
- Obstetric bleed due to PPH
- Hb 3gms, ITU support and resuscitated.

AR

- Retrospective history– bled after dental extraction, menorrhagia
- Had always been iron deficient

- Family history- diagnosis of VWD/bleeding disorder

AR

•VWF:Ag	55.2 iu/dl	(45-160)
•VWF: Ac	36.0 iu/dl	(54-202)
•VWF: CB	28.1 u/dl	(38-245)
• F VIII:C	88.0 iu/dl	(65-170)
•Blood group	A Rh pos	
•Platelet	117 x10^9/L	
•PFA – 100	>300 both cartridges	

Von Willebrand Disease

Overview

- Bleeding disorder
- 1 in 100
- Separated into three subtypes
- Can be Autosomal dominant or recessive

Von Willebrand Disease
Subtypes

- Type 1
  - –Deficiency in Von Willebrand Factor (VWF)
- Type 2
  - –Qualitative abnormalities
- Type 3
  - –Absence of VWF

Von Willebrand Disease
Diagnostic Features

- Type I
  - –Prolonged APTT
  - –Reduced VWF:Ag, VWF:RCo, VWF:CBA
- Type III
  - – Very prolonged APTT
  - – Very Low / Undetectable VWF:Ag, VWF:RCo, VWF:CBA

Platelet Type (Pseudo) VWD

- First described by Weiss in 1982

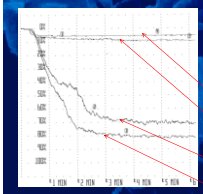
–Weiss, H. J., Meyer, D., Rabinowitz, R., Pietu, G., Girma, J.-P., Vicic, W. J., Rogers, J.: **Pseudo-von Willebrand's disease: an intrinsic platelet defect with aggregation by unmodified human factor VIII-von Willebrand factor and enhanced adsorption of its high molecular weight multimers.**
[illegible] -333, 1982

- Moderate Bleeding
- Normal or reduced platelet count
- Gly233Val or Met239Val in GPIbα
- Distinguished by mixing studies

Platelet Aggregation Profiler (PAP) 4

Born GVRF Aggregation of blood platelets by ADP and its reversal
Nature 1962; 194: 927 - 929 1

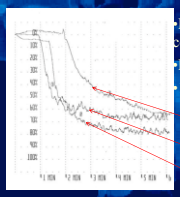
Platelet Aggregation



- Standard Platelet Rich Plasma (PRP)
- Low does ristocetin (0.5g/dl)

ET
Control
DT
AT

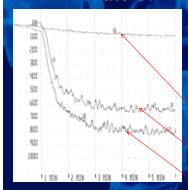
Platelet Mixing Studies



- Patients platelets suspended in control plasma
- Platelet count adjusted
- 1.25g/dl Ristocetin

ET
DT
AT

Platelet Mixing Studies



- Patients platelets in control plasma
- Low dose ritocetin (0.5g/dl)

ET
AT
DT

AR

- Platelet aggregation by aggregometry: Normal
- Ristocetin Induced Platelet Activation (RIPA) positive (PRP with various doses of ristocetin)
- patients platelets/control plasma + ristocetin
- control platelets/patients plasma + ristocetin
- Platelet type VWD
- genetic test: Heterozygous for native glycine substituted to valine at codon 233 in GPIbα
- Pregnant again--- Management

DT Sister

•PFA	Both cartridges >300	
•FVIII	60 iu/dl	(NR 65-170)
•VWF: Ag	38.5iu/dl	(NR 45-160)
•VWF: RCo	23iu/dl	(NR 54-202)
•VWF: CB	32.9 iu/dl	(NR 38-245)
•Plt Count	207 x 10^9/l	(NR 150-400)
•Multimers	Normal low/intermediate Absent high	

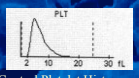
ET (Niece)

•PFA	ADP 186 sec EPI 130 sec	(NR 62-100) (NR 85-165)
•FVIII	80.7 iu/dl	(NR 65-170)
•VWF: Ag	62.5 iu/dl	(NR 45-160)
•VWF: RCo	41 iu/dl	(NR 54-202)
•VWF: CB	66.7 iu/dl	(NR 38-245)
•Plt Count	285 x 10^9/l	(NR 150-400)

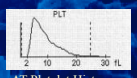
AT (Niece)

- PFA Both cartridges >300
- FVIII 70 iu/dl (NR 65-170)
- VWF: Ag 37.0 iu/dl (NR 45-160)
- VWF: RCo 17 iu/dl (NR 54-202)
- VWF: CB 32.8 iu/dl (NR 38-245)
- Plt Count 182 x 10^9/l (NR 150-400)
- Multimers Normal low/intermediate
  Absent high

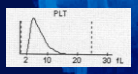
Platelet Sizes



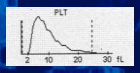
Control Platelet Histogram



AT Platelet Histogram



ET Platelet Histogram



DT Platelet Histogram

Appendix 8 'Platelet case study' presented at IBMS congress 2009

Platelet Case Study

Presentation to:
IBMS Congress, Birmingham 2009

Presented by
David Gurney MSc CSci FIBMS

Guy's and St. Thomas' Hospital NHS

Patient History

- Referral from Jordan
- 7yr old male
- history of intermittent petichiae,
- previous macrothrombocytopenia ?BSS

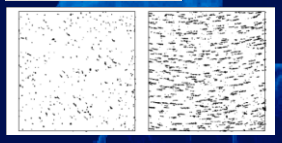
Initial testing

- Full Blood Count
- PFA
- Collagen Epinephrine 162 secs
- Collagen ADP 208 secs
- VWF Screen

Initial testing

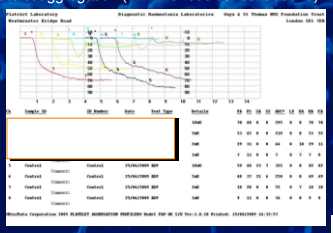
- Impact-R

Surface coverage: 2.6 (>7.5)
Aggregate size: 32 (>25)



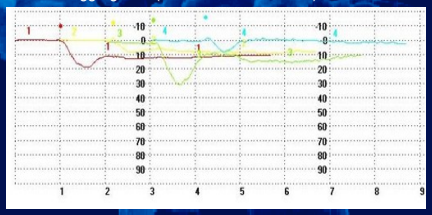
Initial testing

- Platelet aggregation (ADP Various concentrations)



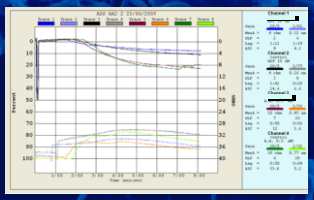
Initial testing

- Platelet aggregation (ADP low concentration)



Further testing

- Whole blood aggregation (High Dose ADP)



Further testing

- Whole blood aggregation (Low Dose ADP)

Appendix 9: 'Platelet analysis; send in the clones' presented at IBMS congress 2011

University Hospitals Bristol NHS
NHS Foundation Trust

Platelet analysis: send in the clones

David Gurney MSc CSci FIBMS
Advanced Biomedical Scientist/Platelet Specialist
Department of Coagulation
8th Floor, Queens Building
Bristol Royal Infirmary
Upper Maudlin Street
Bristol BS2 8HW

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Start with a question

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- Does the type of clone used to raise a monoclonal antibody have a bearing on its efficacy?

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Literature search

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NHS Foundation Trust

Pubmed search found one article comparing clones for flow cytometric analysis

Curvers, J. et al., 2008. Flow cytometric measurement of CD62P (P-selectin) expression on platelets: a multicenter optimization and standardization effort. *Transfusion*, 48(7), p.1439-46

STUDY DESIGN AND METHODS: ***The effects of fixation, source and dilution of CD62P antibody, source of immunoglobulin G (IgG) isotypic antibody, and analysis of results were investigated.*** Once the optimal variables were defined, comparative studies were performed at five participating centers. In the final comparative study, eight split PLT concentrates were shipped to the centers, where samples were stained and fixed accord-ing to the uniform protocol. Analyses were performed using commercially available flow cytometers (BD Bio-sciences and Beckman Coulter).

RESULTS: ***Uniformity between centers could be achieved by using a single clone for CD62P and IgG monoclonal antibody.*** A protocol was selected using fixation with 0.5 percent methanol-free formaldehyde. To increase conformity between flow cytometers, in the analysis of electronic data the thresholds of the isotypic control were set at 0.5 percent for the BD Biosciences and 2 percent for the Beckman Coulter flow cytometers. In the final comparative study, the 95 percent confi-dence intervals (CIs) for CD62P ranged between 8 and 21 percent in fresh and 20 to 40 percent in 8-day-old PLT concentrates.

There was considerable variation between the reported percentages of CD62P- positive PLTs.

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Aim:

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•Establish optimal concentration of available CD61-PE monoclonal antibodies (mAbs) for use on platelets in citrated peripheral blood specimens.

•This concentration will then be used to test each mAb, alongside the Stago kit, on normal adult and paediatric citrated PB specimens.

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Methods

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Brief overview:

• Prepare serial dilutions of all mAbs to be tested, starting at 30ug/ml. The final volume of antibody should be 50ul in all tubes.
•Add sample
•Incubate in the dark for 15 minutes
•Wash
•Acquire on LSR Fortessa
•Analyse

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Clone choices

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Company	Clone	Isotype	conjugate	PE/Ig ratio	cat no	lot	conc
Serotec	Y2/51	IgG1	PE	not stated	MCA2588PE	0307	Not stated. Lyophilized. Recommend add 1ml, use 10ul per test. Probably 100ug/ml
Serotec	PM6/13	IgG1	PE	not stated	MCA728PE	0304	Not stated. Lyophilized. Recommend add 1ml, use 10ul per test. Probably 100ug/ml
Coulter	S221	IgG1	PE	0.5-1.5	PN IM3605	16	Not stated. 20ul per test of 500,000 cells
R&D Systems	256809	IgG2a	PE	not stated	FAB2266P	LNP02 09101	10ug/ml

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Running platelets on flow

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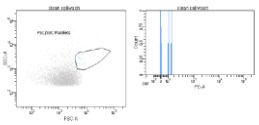
– Be aware of noise levels



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Running platelets on flow

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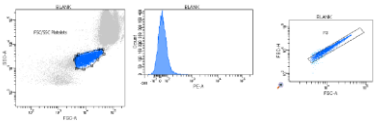
– Be aware of background fluorescence levels of the cells you are looking at



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Running platelets on flow

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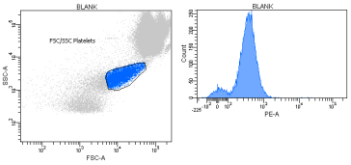
– Be aware of changes induced by processing



Clone differences – Interim data

University Hospitals Bristol NHS NHS Foundation Trust

- Median fluorescence intensity and light scatter parameters measured –

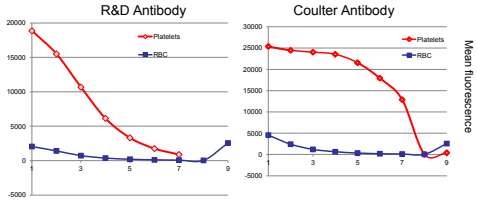
ul mAb | Median Fluorescence Intensities

	R&D	Coulter	Serotec 0307	Serotec 0304
100	18855	25383	9117	15327
50	15498	24469	7163	10695
25	10671	24039	4985	6753
12.5	6144	23536	3165	3995
6.25	3313	21558	1873	2210
3.125	1752	17941	1111	1173
1.5625	884	12878	605	629
0	22	22	0	0

CD30 100ul	405

Results

University Hospitals Bristol NHS NHS Foundation Trust



Results

University Hospitals Bristol NHS NHS Foundation Trust

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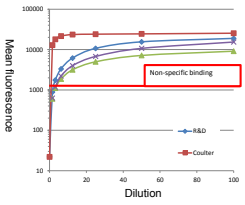
0	22	22	0	0



Clone differences: Interim data 2

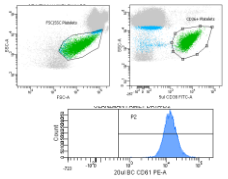
University Hospitals Bristol NHS NHS Foundation Trust

- We found that one clone resulted in brighter signal than the others, irrespective of concentration. (Coulter)
- We found that the forward light scatter was affected by some clones – the effect was reduced at 12.5 ul, but the intensity of the signal then quite low and not suitable for measuring loss of an antigen.

Testing patients

University Hospitals Bristol NHS NHS Foundation Trust

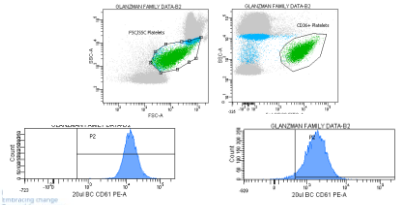
- Here's an example of the CD61 chosen together with CD36, used in the final assay



Testing patients

University Hospitals Bristol NHS NHS Foundation Trust

- And next, a case of Glanzmanns Thrombasthenia (GT)



Testing patients

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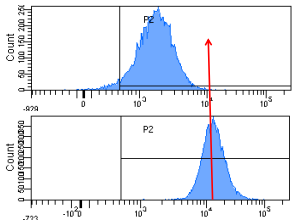
•Current practice in many laboratories is to run a normal control next to a query.

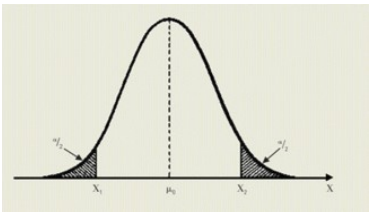
•While this would certainly be adequate for finding clear-cut GT cases, a more exact, definite value of reduction of antigen expression requires a reference range for normal and non-glanzman disease controls.

•Frequently, GT queries will be samples form children and an age-appropriate reference range should be aimed for.

Testing patients



Reference Ranges



~~NORMAL~~

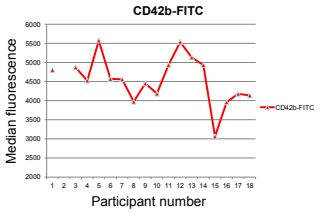
Reference Ranges

- As a new assay has being developed new reference ranges are needed.
- 17 subjects have been recruited so far
- 10 females 5 males
- Average age 35 years old

Need more subjects to give range more power

Reference Ranges

Expression of CD42b (GPIb) varies in a cohort of 17 ‘normal's’ .



Appendix 10: Platelet disorders: Test selection, reporting and result interpretation SOP for St Thomas' hospital

Centre for Haemostasis and Thrombosis

Standard Operating Procedure

Document Number: **HT-SOP-HSP-013**

Document Title: **Platelet Disorders: Test selection, reporting and result interpretation**

DOCUMENT DETAILS & AUTHORISATION

Version Number:	01.0	
Issue Date:		
Review Date:		
Author	David Gurney	
Authorised By:	Dr Gary Moore	Date:
	Dr Savita Rangarajan	Date:

REVISION CHANGES

Version Number	Change Details	Date

Authorisation Expires Two Years from Date of Revision Approval

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Introduction

Background

Platelet disorders are notorious difficult to diagnose. Most laboratory testing is qualitative and although some thought has been put into quantification of these tests there is still a broad spread of reporting styles. *(I. Jennings, et al. (2008). `Platelet function testing: practice among UK National External Quality Assessment Scheme for Blood Coagulation participants, 2006.'* Journal of clinical pathology ***61****(8):950-954, K. A. Moffat, et al. (2005). `Variability in clinical laboratory practice in testing for disorders of platelet function: results of two surveys of the North American Specialized Coagulation Laboratory Association.'.* Thrombosis and haemostasis ***93****(3):549-553,)* This SOP is to be used to help with interpretation of a range of platelet assays and hopefully bring some standardisation to our reporting regime.

It is not a replacement for the method SOPs.

Guidelines

Until very recently the last official set of guidelines for platelet testing were published in the Journal of Clinical Pathology in 1988 (*Journal of clinical pathology, Vol. 41, No. 12. (December 1988), pp. 1322-1330*). These had a clinical update in 2006 when the UKHCDO and BSHT published a joint document (*British Journal of Haematology, Vol. 135, No. 5. (December 2006), pp. 603-633*)

Committee for Laboratory Standards Institute (CLSI)

The CLSI of America published guidelines for aggregation in October 2008. A copy is available in the department.

International Society for Thrombosis and Haemostasis (ISTH)

At the 2009 Special Scientific Committee (SSC) meeting of the ISTH in Boston the minutes show that guidelines for light transmission aggregometry (LTA) were high on the committees' agenda.

British Committee for Standardisation in Haematology (BCSH)

The BCSH guidelines were mentioned in the minutes of the ISTH SSC 2009. Dr Paul Harrison is involved with this and we hope to hear from him as soon as they are available.

Testing

Platelet Counting

Automated Full Blood Count

When running a full blood count (FBC) the platelet count will be shown. Some disorders are purely qualitative, in that the patient has insufficient platelets to perform their given function. This will be highlighted by a low platelet count. Other disorders (such as Glanzmann's thrombasthenia) give a normal FBC platelet picture. Other information that can be gleaned from the FBC is the size. By looking at the size histogram plots (See Figs 1 & 2 below) we can see the distribution of platelet sizes. Some diseases such as Bernard Soulier syndrome (BSS) will have a macrothrombocytopaenic picture (low numbers of large platelets)

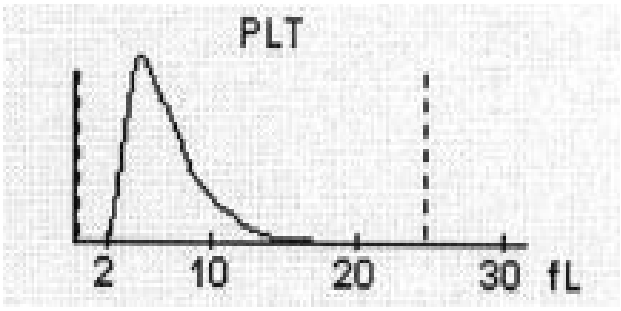


Fig 1: Normal platelet histogram from a Coulter LH750

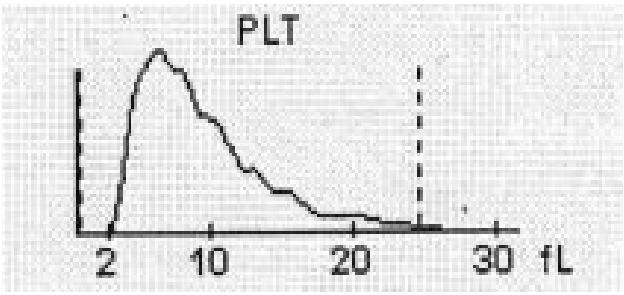


Fig 2: Abnormal platelet histogram showing various sizes and an extended 'tail'

Counting chamber

As the coulter method for counting platelets relies on size and gating, it can be deceived by large platelets in the red cell channel or small red cells appearing in the platelet channel. If this was suspected then a manual platelet count can be performed using an improved Neubauer counting chamber (see Fig 3 below). Whilst counting an assessment of the platelet sizes can also be make by an experienced BMS

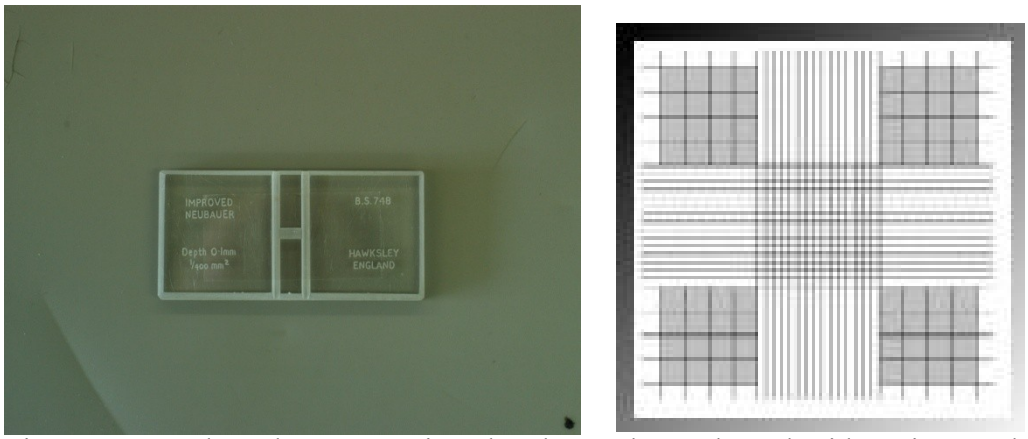


Fig 3: Improved Neubauer counting chamber and an enlarged grid section used for counting platelets

Immuno-platelet count

When platelet counts are done visually or by size, there are inherent risks in the procedure to be overcome. One way of doing this is to count the platelets using platelet specific antibody labelling. The method used in Haematology is based on methodology from *International Council for Standardization in Haematology Expert Panel on Cytometry, International Society of Laboratory Hematology Task Force on Platelet Counting. Platelet counting by the RBC/platelet ratio method. A reference method. American journal of clinical pathology. 2001 March;115(3):460-464.* While this is more accurate it is not without its pitfalls. The antibodies used bind to specific glycoproteins on the platelet, both of which have been well characterised as being deficient in some disease states (see glycoprotein analysis).

Film

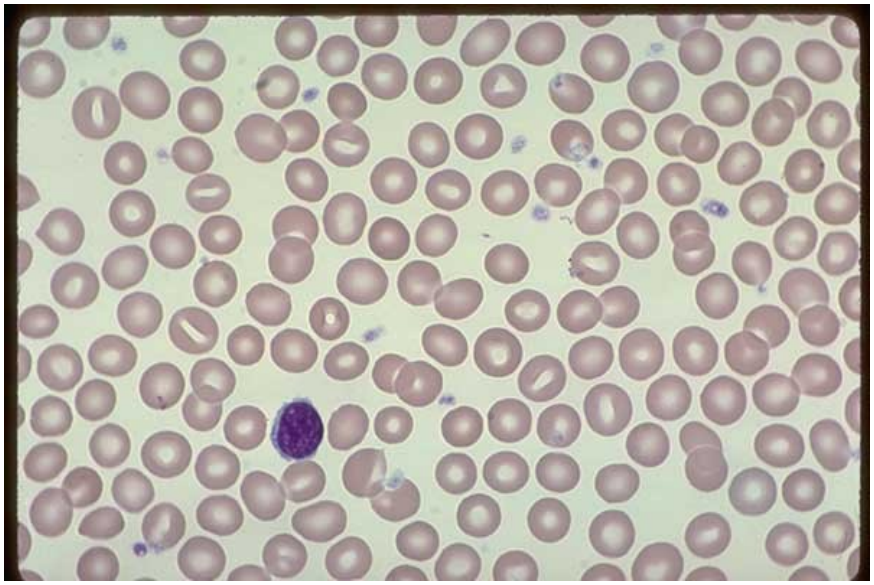


Fig 4: Normal romanovsky stained blood film

Looking at the film should reinforce what has been seen with the platelet histogram data, if this is not the case further investigation is warranted. When looking at the platelets on the slide consider the numbers at a lower power magnification and then scrutinise the platelets more closely under higher power. Questions to ask are:

- Are they all the same size-

a. If they are all normal? Large? Small?

b. If they are not all the same size - are there distinct populations? Various sizes

- What shape are they?
- Are they stained adequately?

a. If the rest of the film is stained adequately and the platelets are not it could be a granule deficiency such a grey platelet syndrome (α- granule deficiency)

PFA-100 (Platelet Function Analyser)

Originally described in *Kundu SK, Heilmann EJ, Sio R, Garcia C, Davidson RM, Ostgaard RA. Description of an in vitro platelet function analyzer-PFA-100. Seminars in thrombosis and hemostasis. 1995;21 Suppl 2:106-112* the platelet function analyser (PFA-100) has come to replace the bleeding time in many laboratories. This analyser uses pressure to recreate the shear forces mimicking movement through the vasculature. To simulate a break in the vasculature collagen is used with either ADP or epinephrine as a stimulate agonist. The time taken for the platelets to form a thrombus and block the capillary, creating and equal opposite pressure to the shear forces is called the 'closure time'. Much was claimed of the PFA-100 when it was introduced over a decade ago. There is no benefit doing a PFA-100 if a collagen/vasculature based bleeding disorder, such as hereditary telangiectasia, is suspected.

However it is now best used as a screening test and regardless of whether it is prolonged or not, if there if sufficient clinical evidence to go onto to perform platelet aggregation, then this is what should be done.

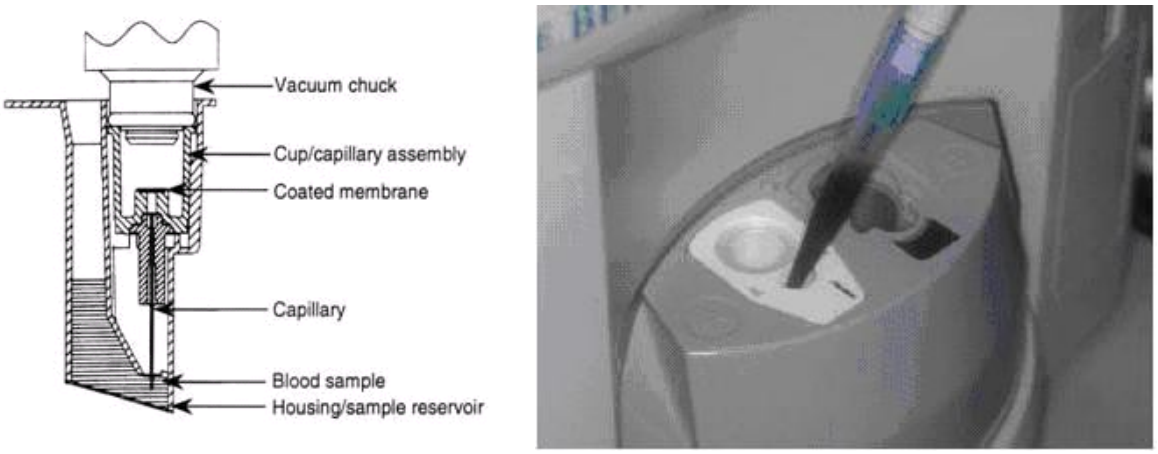


Fig 5: Cut away of a PFA-100 cartridge and sample being loaded into the analyser. Images courtesy of Sysmex UK

	Collagen ADP Cartridge Closure time (seconds)	**Collagen Epinephrine** Cartridge Closure time (seconds)
Normal Range	62 - 100	85 - 165
COX deficiency	N	P
Aspirin Ingestion	N	P
Glanzmann's Thrombasthenia	P	P
Thromboxane receptor defects	N	P
P_2Y12 receptor defects (Clopidogrel resistance)	N/P	N/P
VWD	P	P
***Due to the Hb and PCV constraints of this assay Bernard Soulier Syndrome, the MYH9 disorders & grey platelet syndrome cannot be diagnosed by PFA as these are macrothrombocytopenias and the platelet count will be too low.**		

Table 6: PFA100 results. Normal ranges and some responses in common platelet defects.
N= Normal P = Prolonged
Modified from: ***Hayward CPM, Harrison P, Cattaneo M, Ortel TL, Rao AK. Platelet function analyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function. Journal of Thrombosis and Haemostasis. 2006 February;4(2):312-319.***

~However it is now best used as a screening test and regardless of whether it is prolonged or not, if there if sufficient clinical evidence to go onto to perform platelet aggregation, then this is what should be done.~

Platelet aggregation (PA): (HSP-002)

Platelet aggregation (PA)**:** PRP, sometimes known as Born aggregometry (after its inventor *Born GVR. Aggregation of Blood Platelets by Adenosine Diphosphate and its Reversal. Nature. 1962 June;194(4832):927-929.)* or light transmission aggregometry (LTA, after the technique used)

Gustav Born

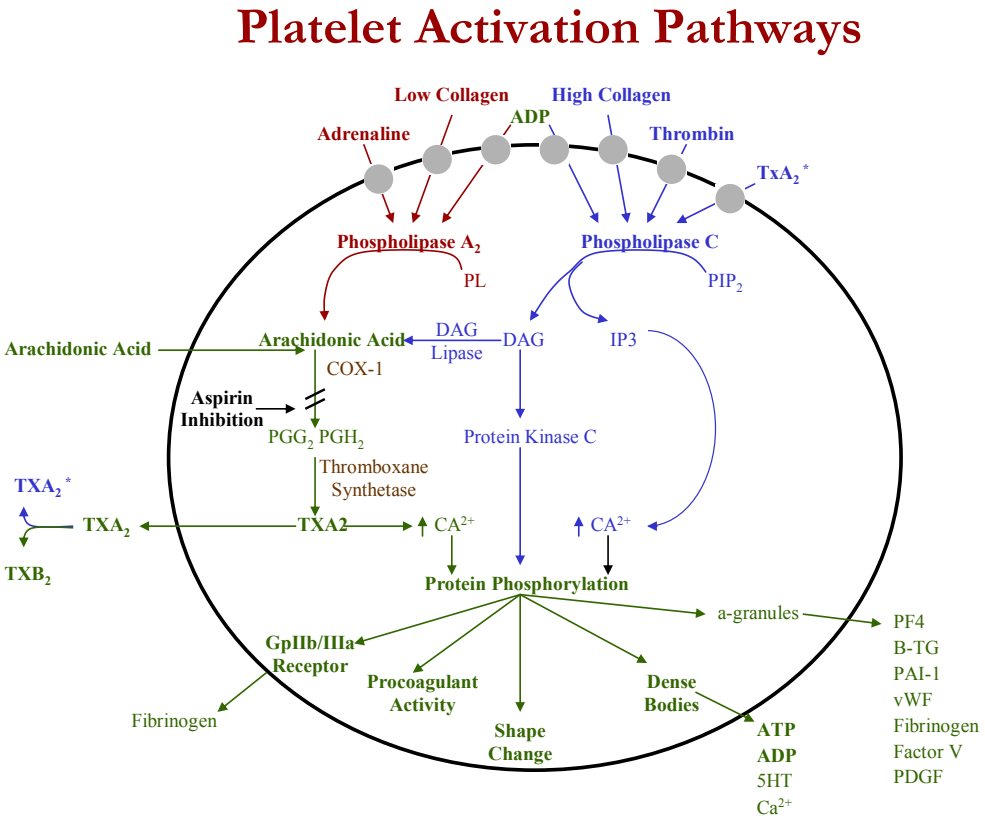


Fig 7: Platelet activation pathways: courtesy of Labmedics/Chronolog

Normal aggregation traces: ADP

ADP is essential for platelet aggregation. It is stored in the delta (δ) or dense granules. When released into the bloodstream it reacts with receptors on the platelet surface. These receptors are named P2Y1, P2Y12 and PX1. P2Y1 in the above diagram is the ADP receptor activating phospholipase C, the process which eventually leads to shape change. P2Y12 interacts with phospholipase A_2

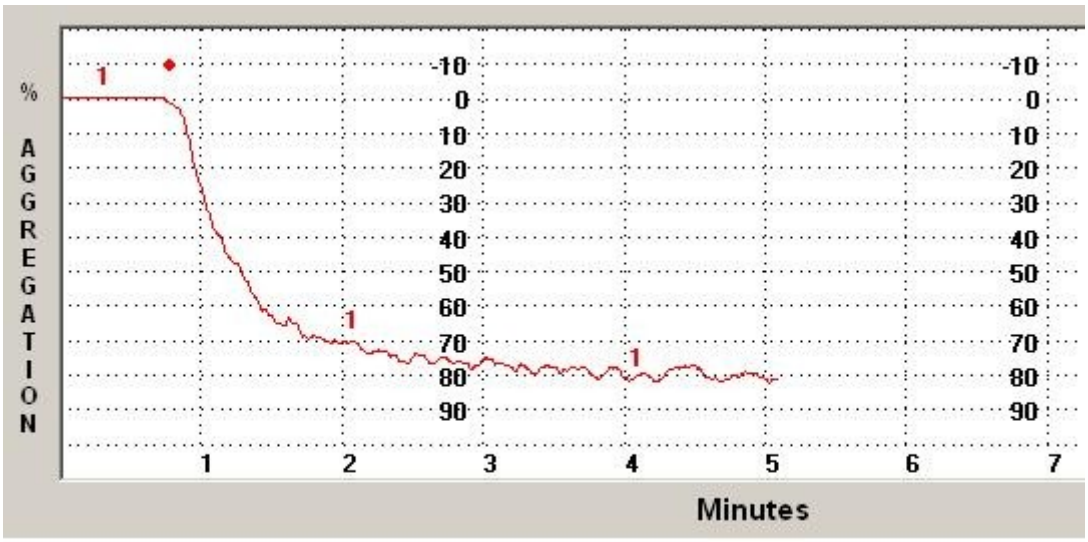


Fig 8: ADP 10µM platelet aggregation trace as displayed on the BioData PAP-8E

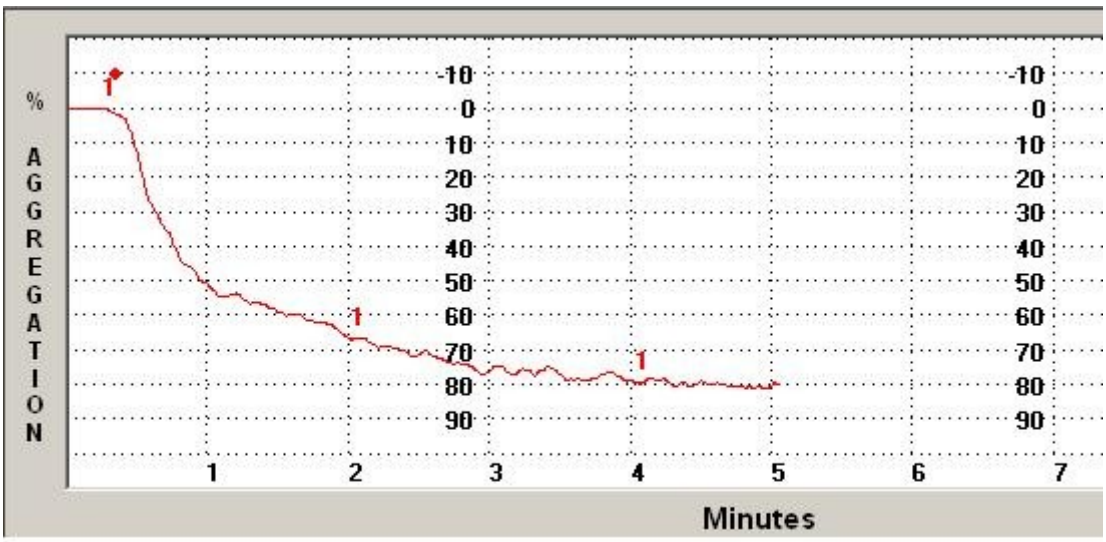


Fig 9: ADP 5µM platelet aggregation trace as displayed on the BioData PAP-8E

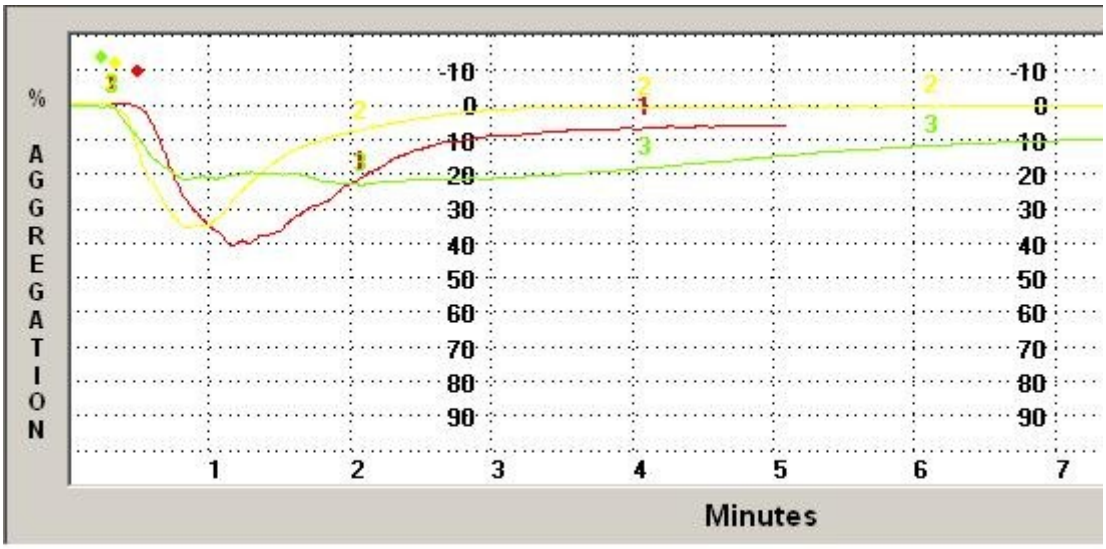


Fig 10: ADP 2µM platelet aggregation traces as displayed on the BioData PAP-8E, showing the variation in the 'biphasic' response

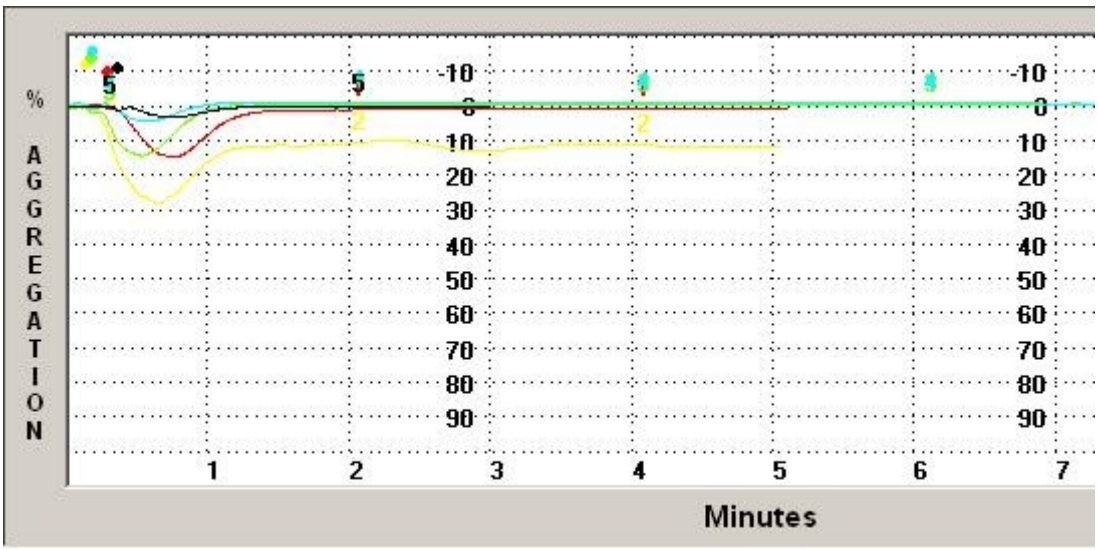


Fig 11: ADP 1µM platelet aggregation traces as displayed on the BioData PAP-8E, showing the variation in the 'biphasic' and 'flat-line' response

Normal aggregation: Collagen

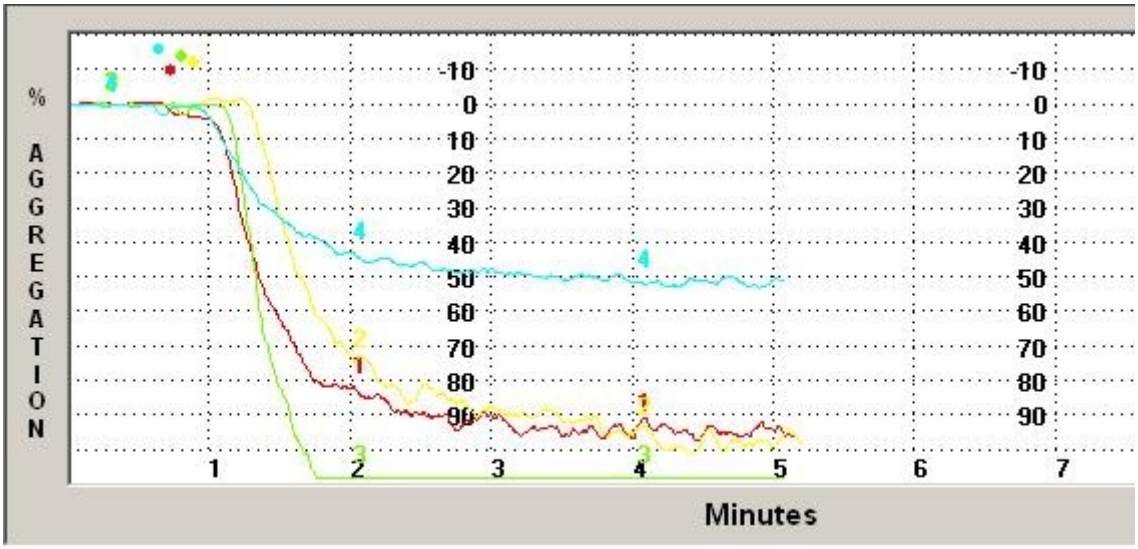


Fig 12: Collagen 20g/L platelet aggregation traces as displayed on the BioData PAP-8E

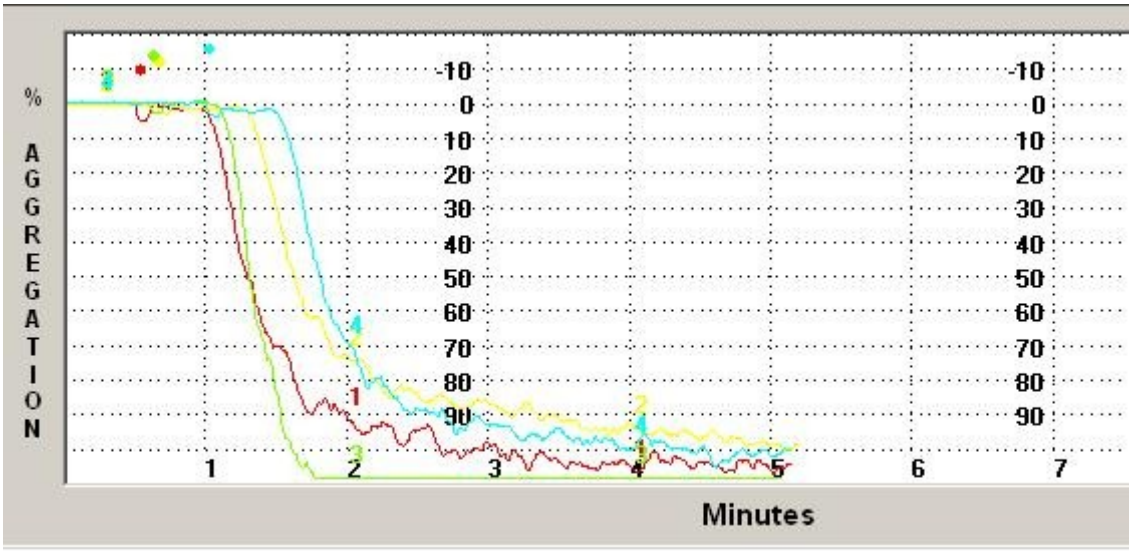


Fig 13: Collagen 5g/L platelet aggregation traces as displayed on the BioData PAP-8E

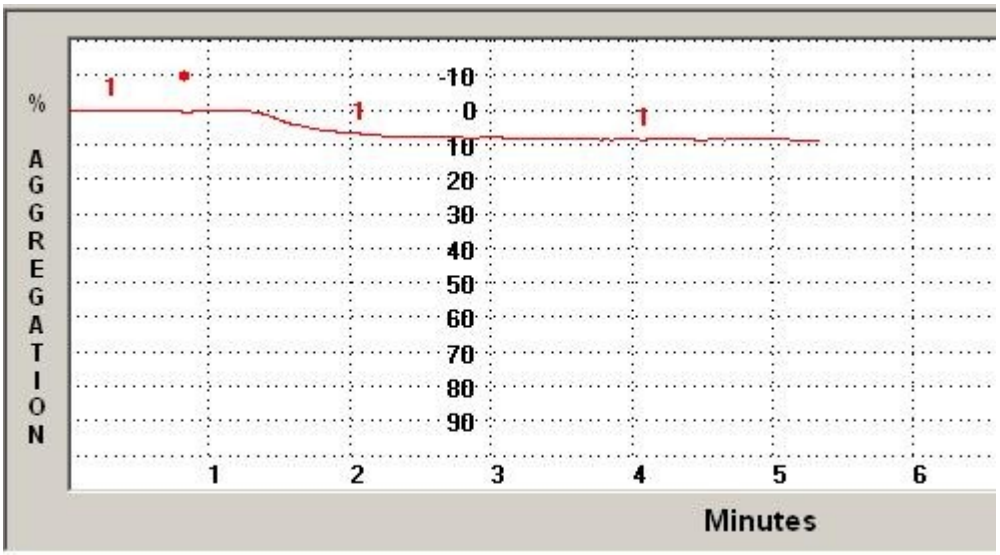


Fig 14: Collagen 1g/L platelet aggregation traces as displayed on the BioData PAP-8E

Normal aggregation: Epinephrine

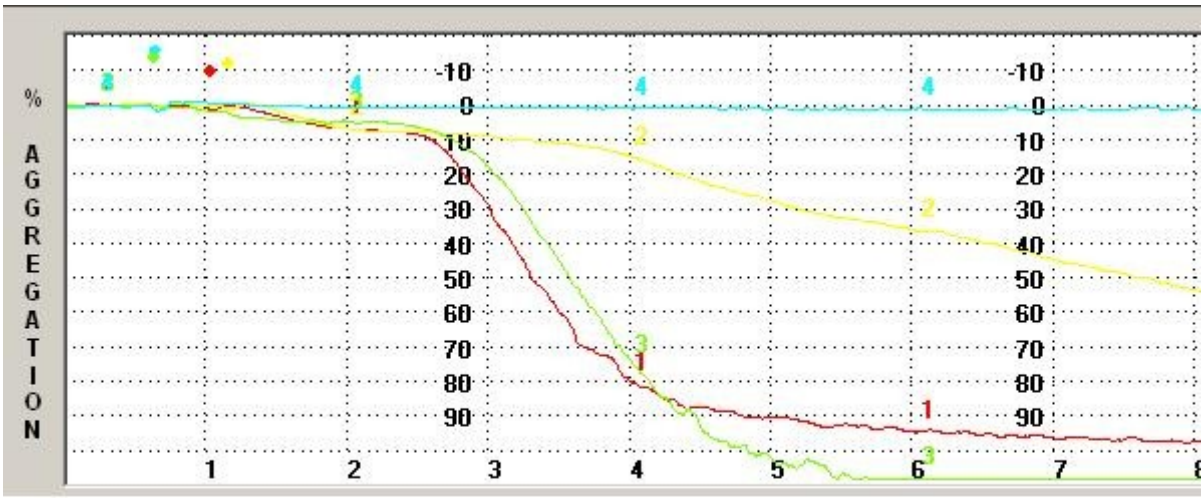


Fig 15: Epinephrine 20µM platelet aggregation traces as displayed on the BioData PAP-8E.

Note the variability of the traces

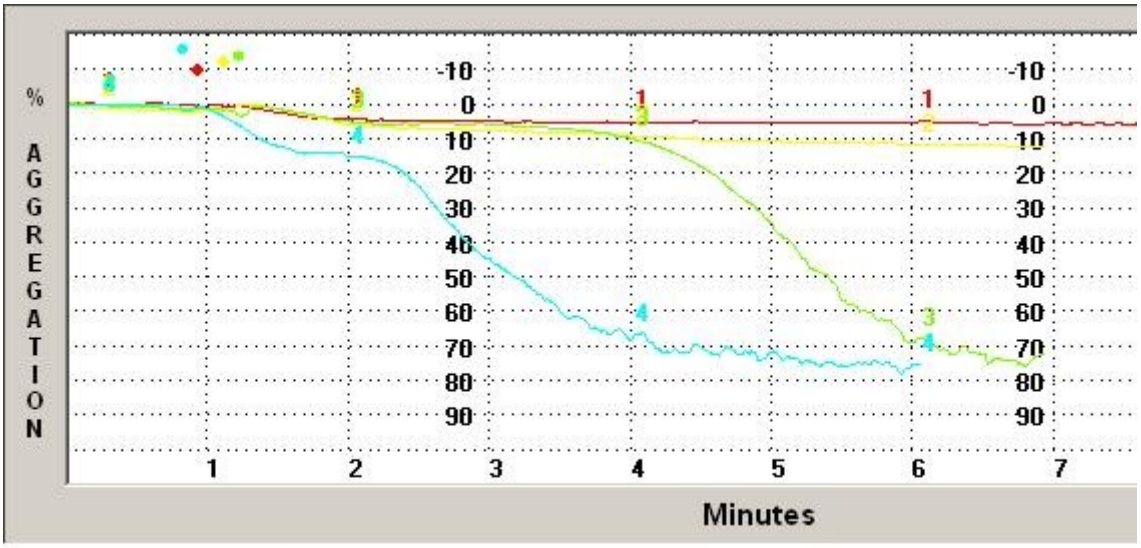


Fig 16: Epinephrine 2µM platelet aggregation traces as displayed on the BioData PAP-8E.

Note the variability of the traces

Normal aggregation: Arachidonate

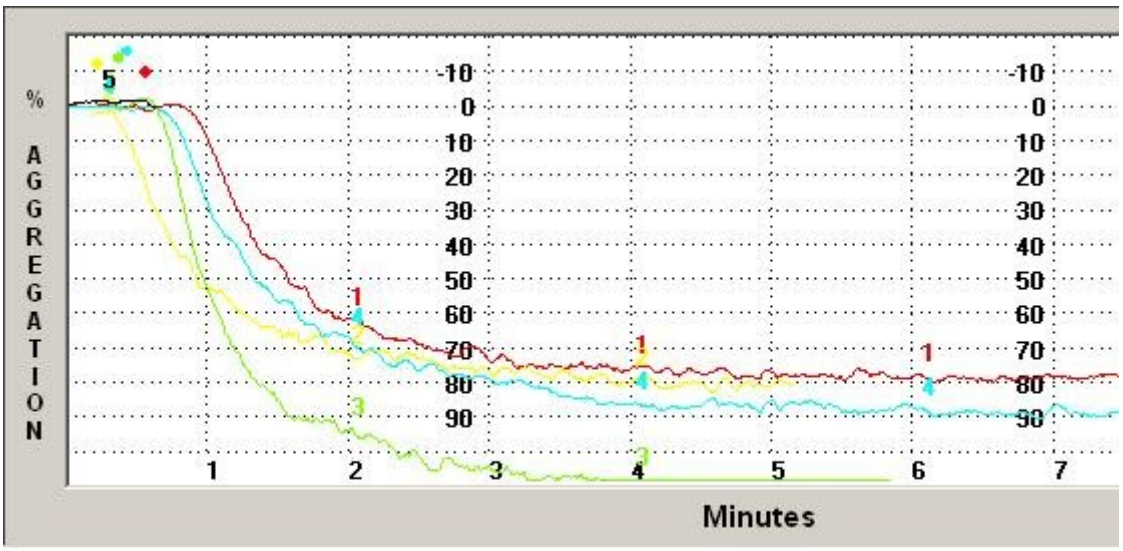


Fig 17: Arachidonate, also known as aracidonic acid platelet aggregation traces as displayed on the BioData PAP-8E.

Normal aggregation: Ristocetin

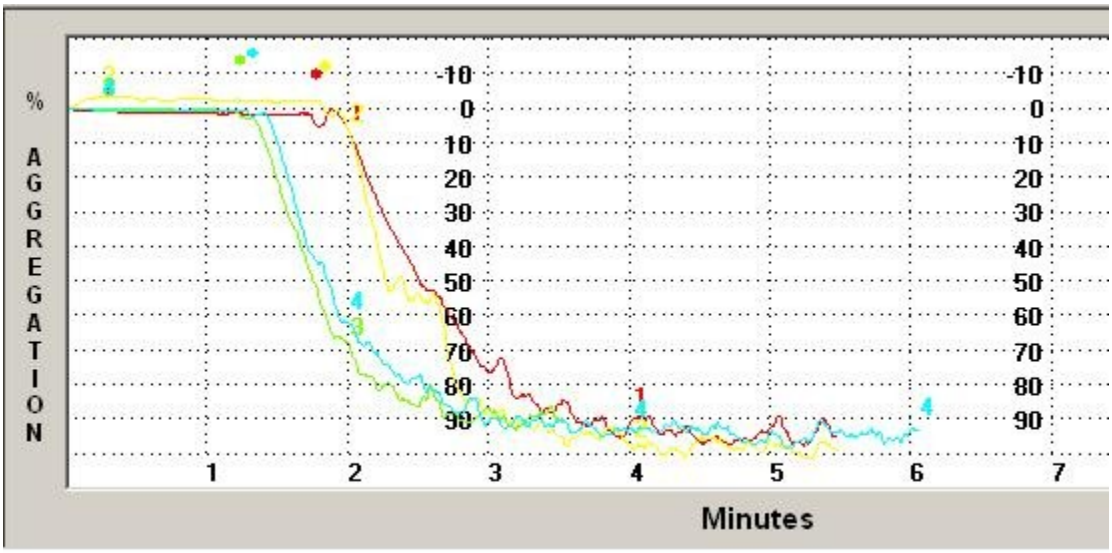


Fig 18: Ristocetin 1.25g/L platelet aggregation traces as displayed on the BioData PAP-8E

Normal aggregation: Thrombin receptor agonist peptide (TRAP)

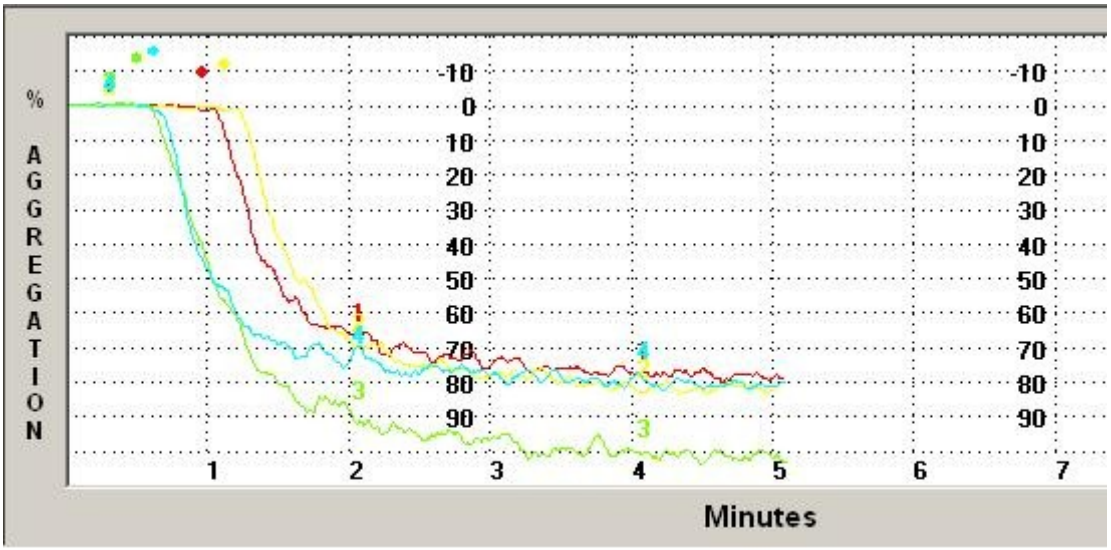


Fig 19: Thrombin receptor agonist peptide (TRAP) 20µM platelet aggregation traces as displayed on the BioData PAP-8E

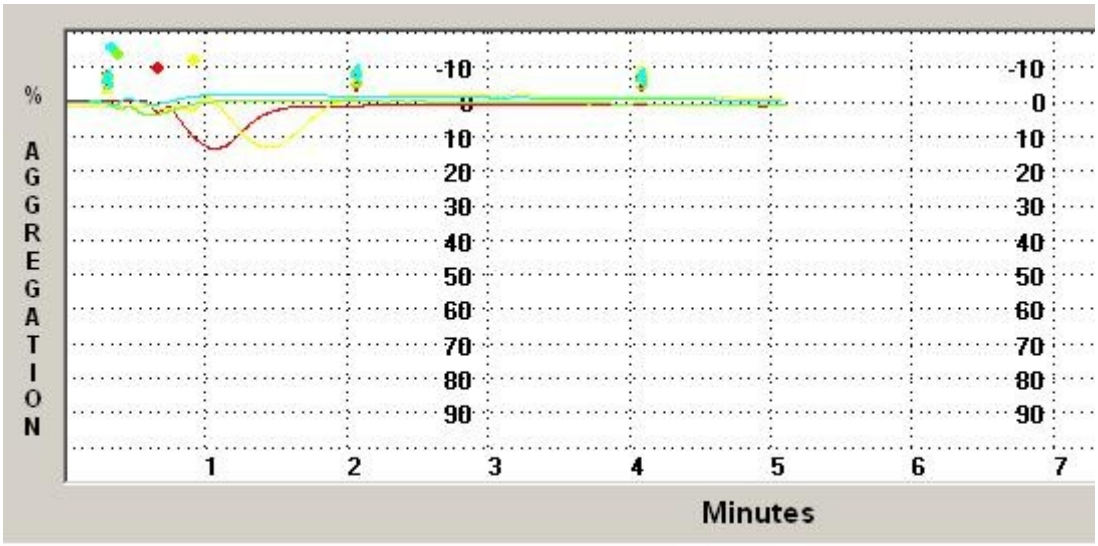


Fig 20: Thrombin receptor agonist peptide (TRAP) 10µM platelet aggregation traces as displayed on the BioData PAP-8E

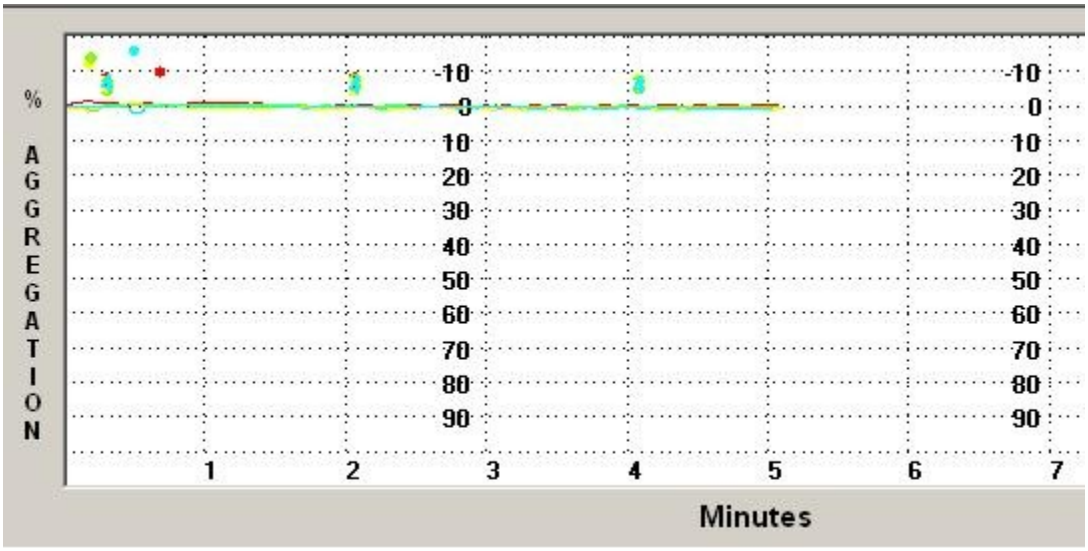


Fig 21: Thrombin receptor agonist peptide (TRAP) 1µM platelet aggregation traces as displayed on the BioData PAP-8E

Normal aggregation: U46619

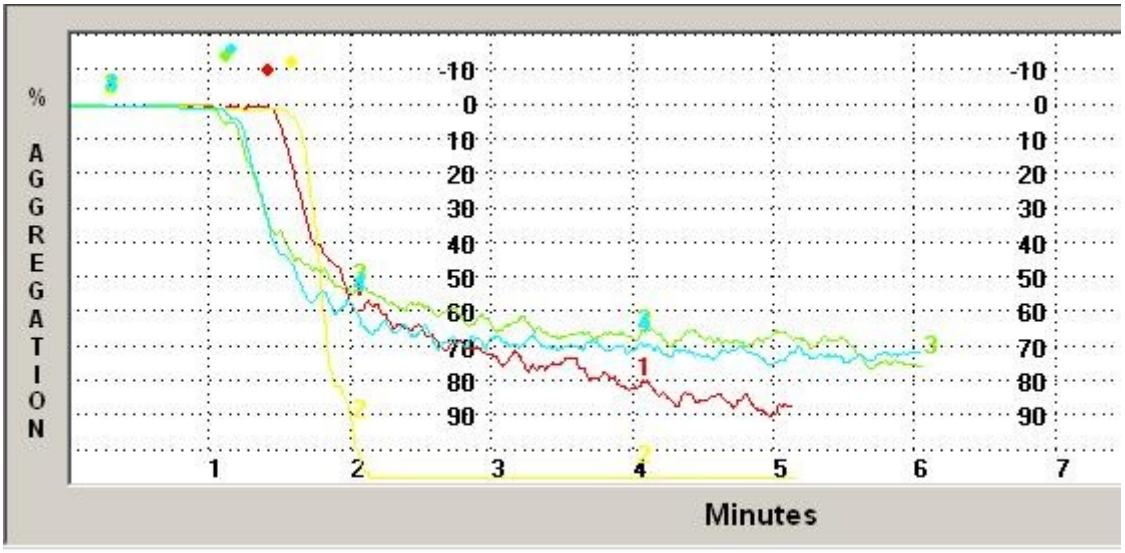


Fig 22: U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) 100µM platelet aggregation traces as displayed on the BioData PAP-8E

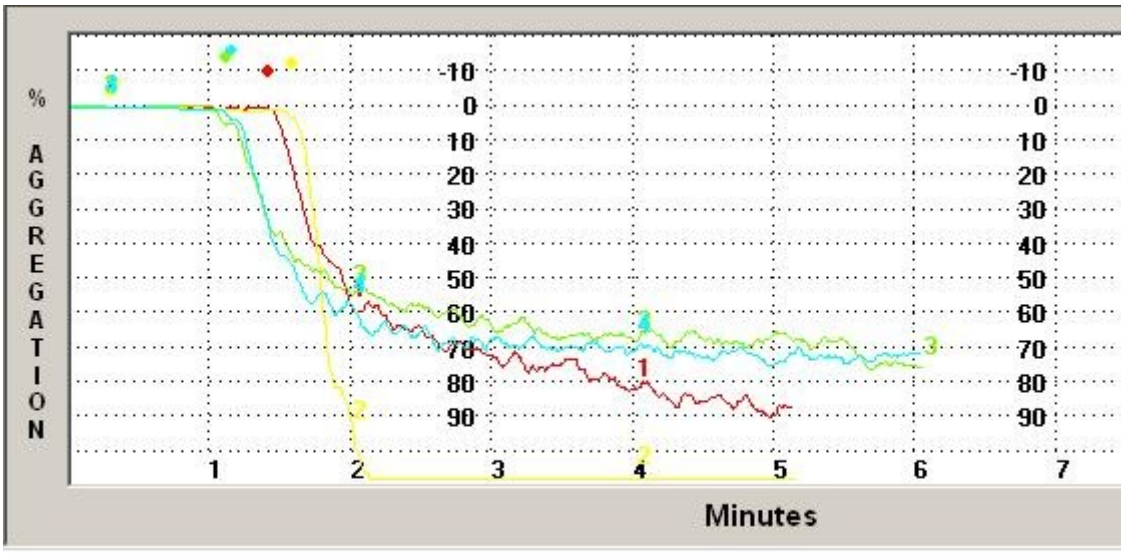


Fig 23: U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) 50µM platelet aggregation traces as displayed on the BioData PAP-8E

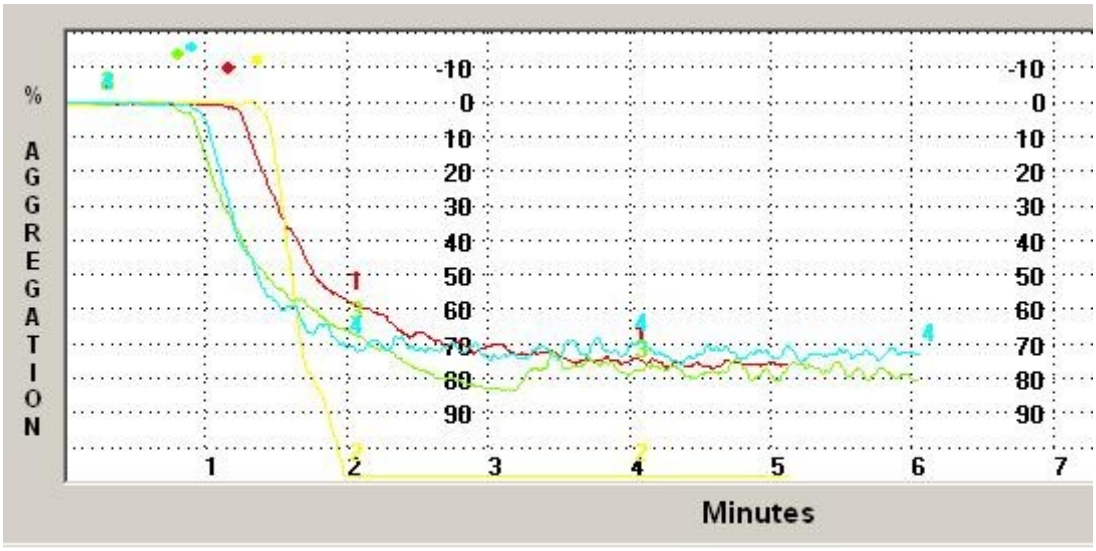


Fig 24: U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) 25µM platelet aggregation traces as displayed on the BioData PAP-8E

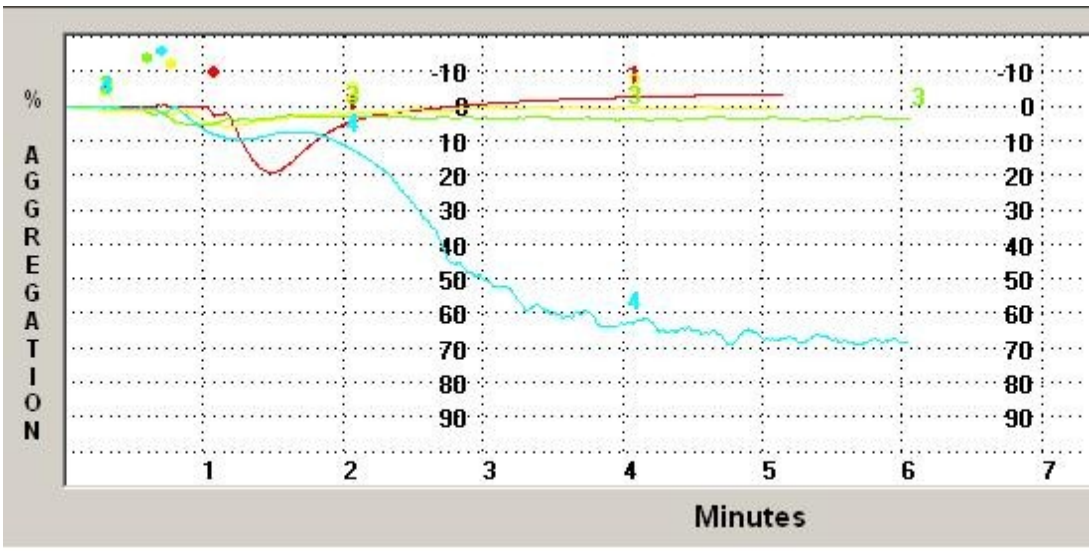


Fig 25: U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) 5µM platelet aggregation traces as displayed on the BioData PAP-8E, showing variation in response at this dosage of the agonist

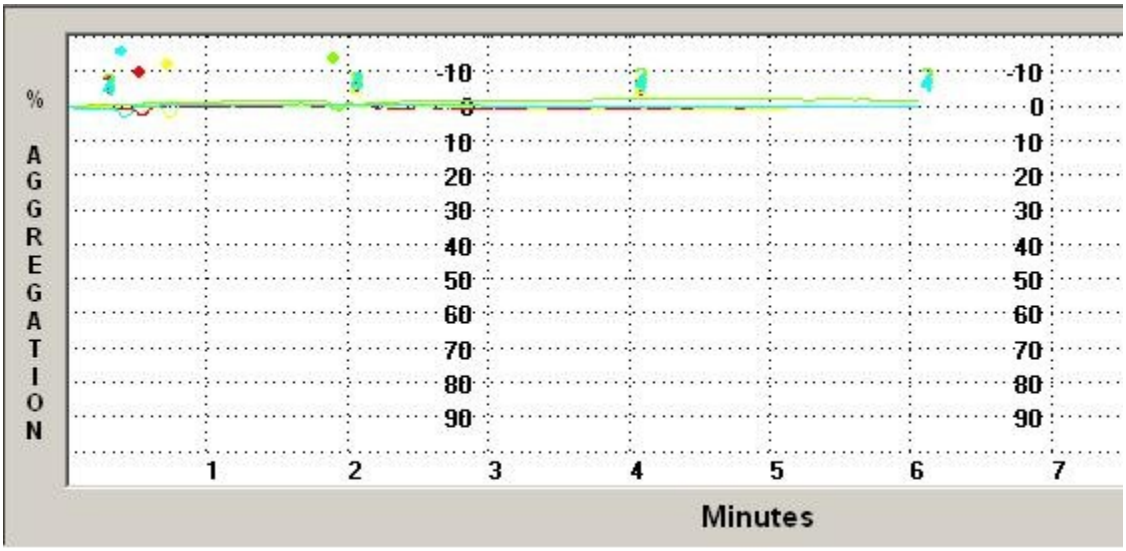


Fig 26: U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) 500nM (0.5μM) platelet aggregation traces as displayed on the BioData PAP-8E

Interpretation

When looking at platelet traces there are a number of characteristics to consider:

- Agonist response

a. If it responds rapidly at low doses, dilute until a biphasic response is seen

b. If it does not respond increase agonist concentrations until one is observed

- Slope. The slope of the traced line indicates how quickly the platelets are reacting to a given stimulus. A shallow slope is a slow reaction and could indicate a breakdown in the mechanisms of the platelet response.
- Maximum aggregation.

a. How far down does the slope go?

b. How far should it go?

c. How does it compare to the control? A reduced maximum aggregation indicates a reduction in the platelets response to an agonist.

- Pattern.

a. Is there a biphasic response?

b. Is it stepped and proceeds to full aggregation?

c. Alternatively does is the trace indicate that the platelet responds then reverses back?

- How far does it reverse? Completely? Partially?

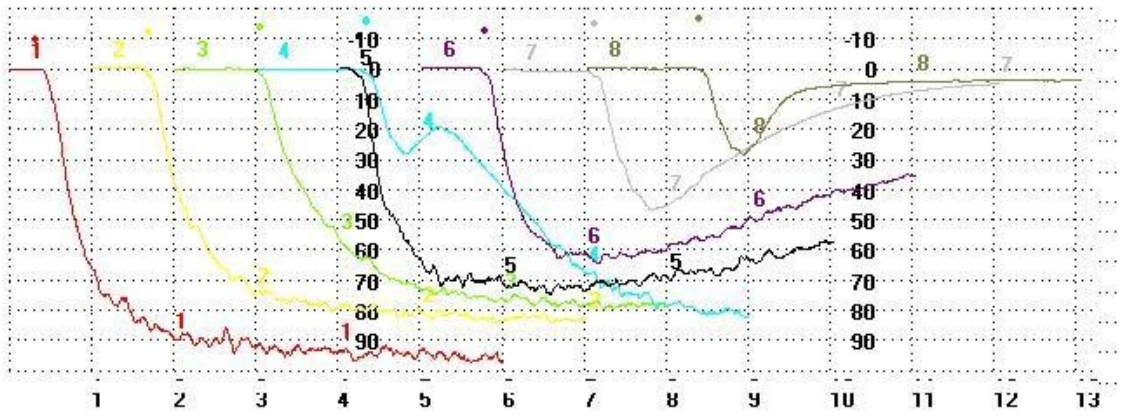


Fig27: Example of ADP traces showing possible storage pool disorder.
Control traces 1 - 4 (10µM, 5µM, 2µM & 1µM) Patient 5 – 8 (10µM, 5µM, 2µM & 1µM)

Many primary haemostatic or platelet disorders present with distinct aggregation patterns. Delta storage pool disorder, which is a lack of delta or dense granules, exhibits a biphasic response with ADP, the stronger doses of agonist giving a stepped style of responses (See Fig 27) where as the lower doses give what we refer to as ‘ a biphasic response with full or partial reversal’ (See Fig 27 for examples).

Drugs such as aspirin, clopidogrel (Plavix©), and Prasugrel affect platelet function. Aspirin is an irreversible cyclooxygenase isomer 1 (COX1) inhibitor. In platelet aggregation testing this will manifest as a reduction or absence of response to ADP and Arachidonate, but is restored when U46619 is used. This pattern will also be observed if there is an enzyme problem, often called ‘aspirin like’ deficiency. If neither arachidonate nor U46619 work, the defect is likely to be in the thromboxane receptor.

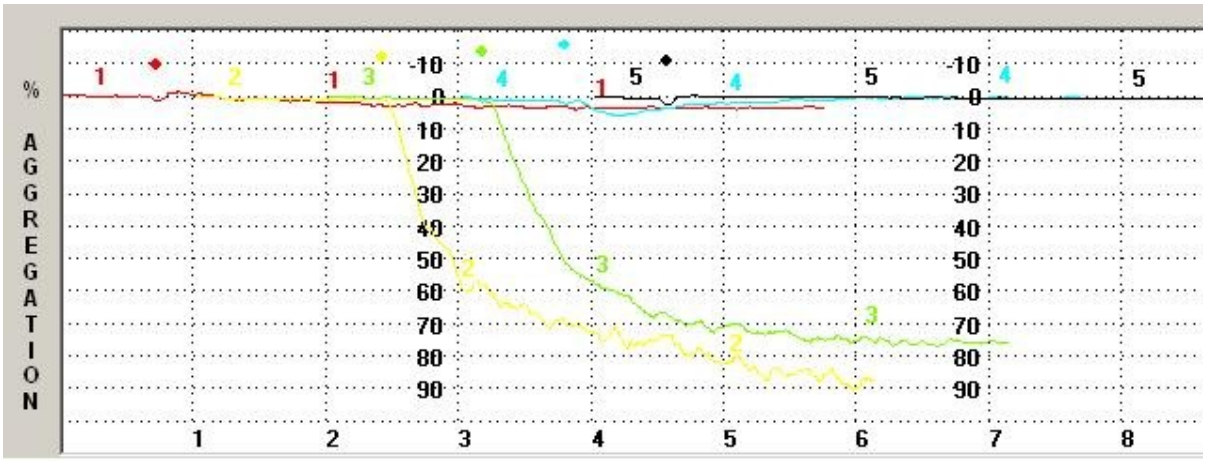


Fig 28: Showing an abnormal arachidonate trace (Line 1 in red) with a normal series of U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) traces (Lines 2-5) The possible diagnosis is an enzyme deficiency (either acquired or genetic)

Clopidogrel and Prasugrel, bind to the P2Y12 receptor so will blunt the ADP response.

Receptor disorders especially glycoprotein (GP) based diseases give a distinct 'combination' pattern. Glanzmann's thrombasthenia, which involves the GPIIb/IIIa receptor is reduced or absent with ADP, collagen, epinephrine, but not with ristocetin. Bernard Soulier syndrome is only reduced with ristocetin, but has other distinguishing characteristics such as macrothrombocytopaenia. If the patient has macrothrombocytopaenia, but responds to ristocetin then an MYH9 disorders such as May-Hegglin, Fechner or Sebastian syndrome would be suspected.

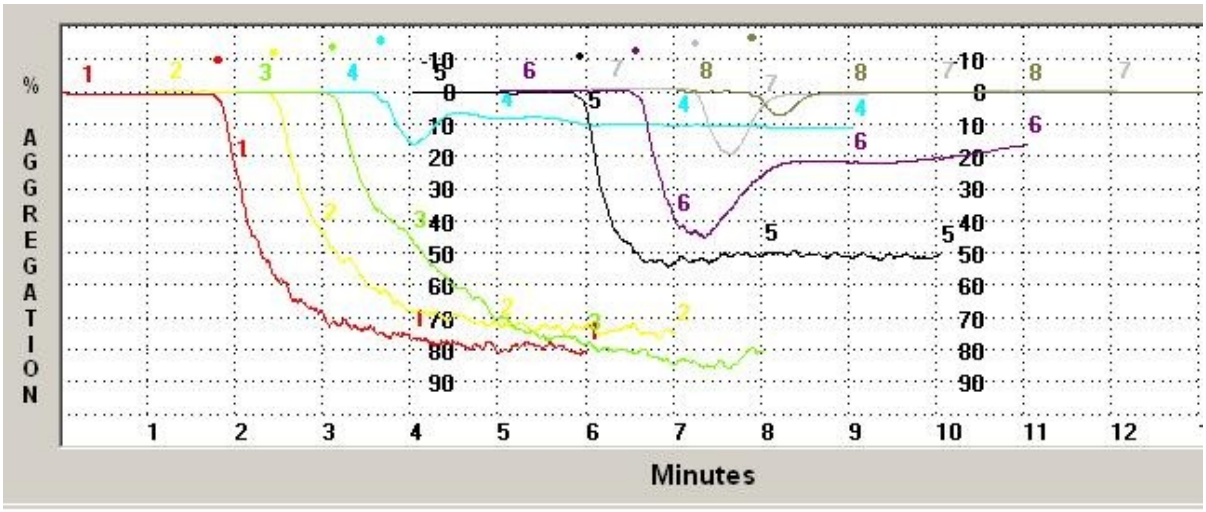


Fig 29: ADP traces showing control (Lines 1-4 10µM, 5µM, 2µM & 1µM) and a patient with possible Ehlers danlos syndrome (Lines 5-8 10µM, 5µM, 2µM & 1µM)

Example of a subjective written report for the above traces (Fig 29):-

Control

Line 1 (Red): Full aggregation (81%)

Line 2 (Yellow): Full aggregation (78%)

Line 3 (Green): Full aggregation (73%)

Line 4 (Cyan): Biphasic aggregation with 18% first phase with partial reversal 10%

Patient

Line 5 (Black): First phase aggregation (52%) with no second phase

Line 6 (Purple): First phase (42%) with reversal (21%)

Line 7 (Silver): First phase (19%) with full reversal

Line 8 (Grey): First phase (5%) with full reversal

Depending on the results of the aggregation, this will dictate the direction of further testing. As above the presence of a first phase with reversal with ADP as an agonist further testing would lean toward granular disorder testing such as nucleotides.

Platelet aggregation (Whole blood): **sometimes known as impedance aggregometry, after the technique used.** *(Cardinal DC, Flower RJ. The electronic aggregometer: a novel device for assessing platelet behaviour in blood. Journal of pharmacological methods. 1980 February;3(2):135-158.)*

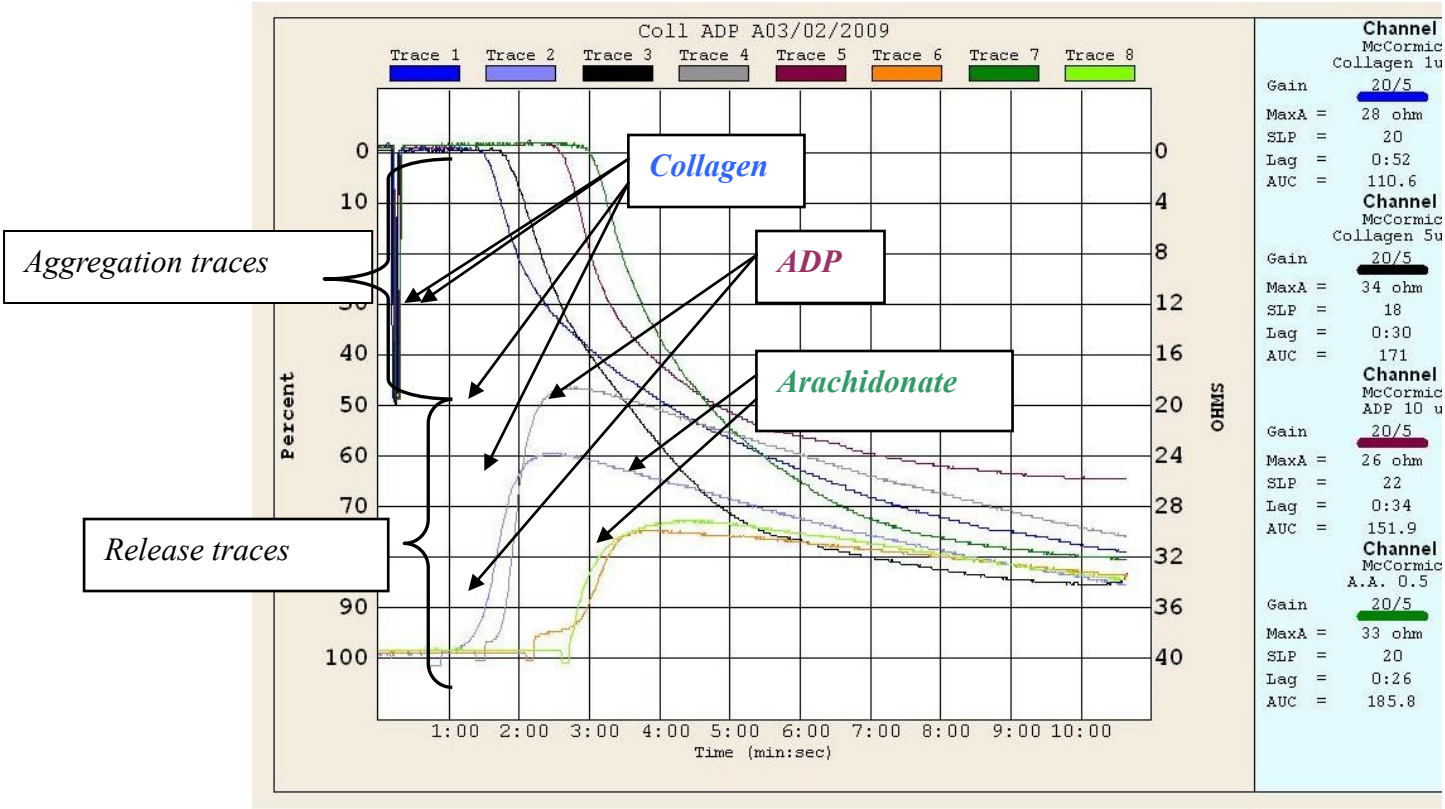
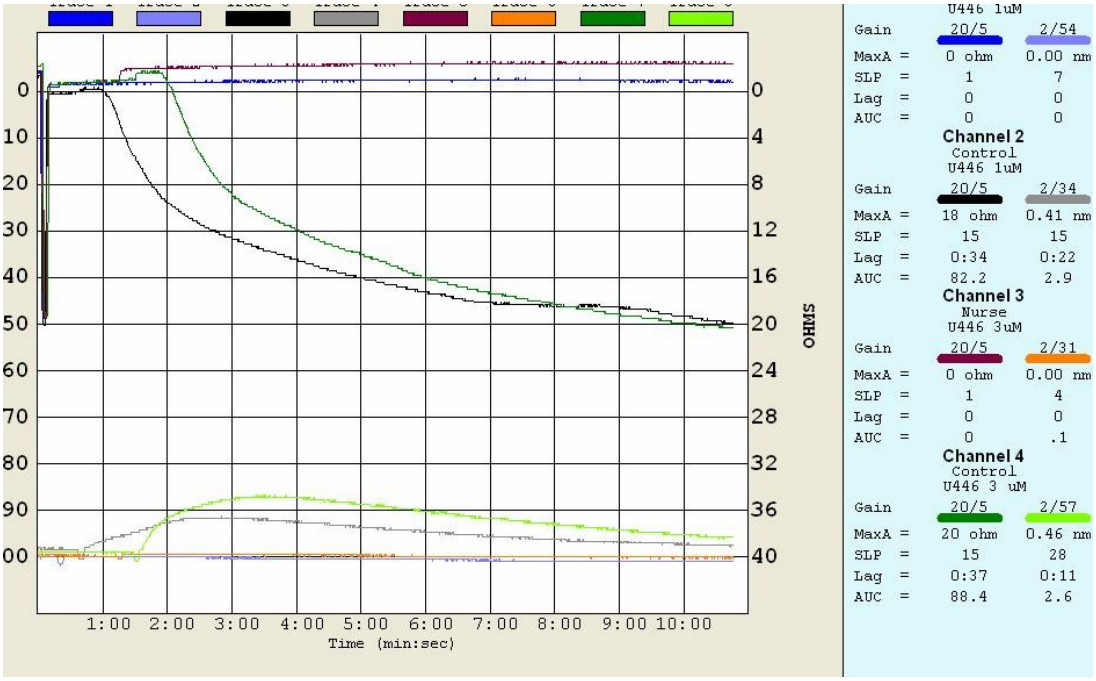
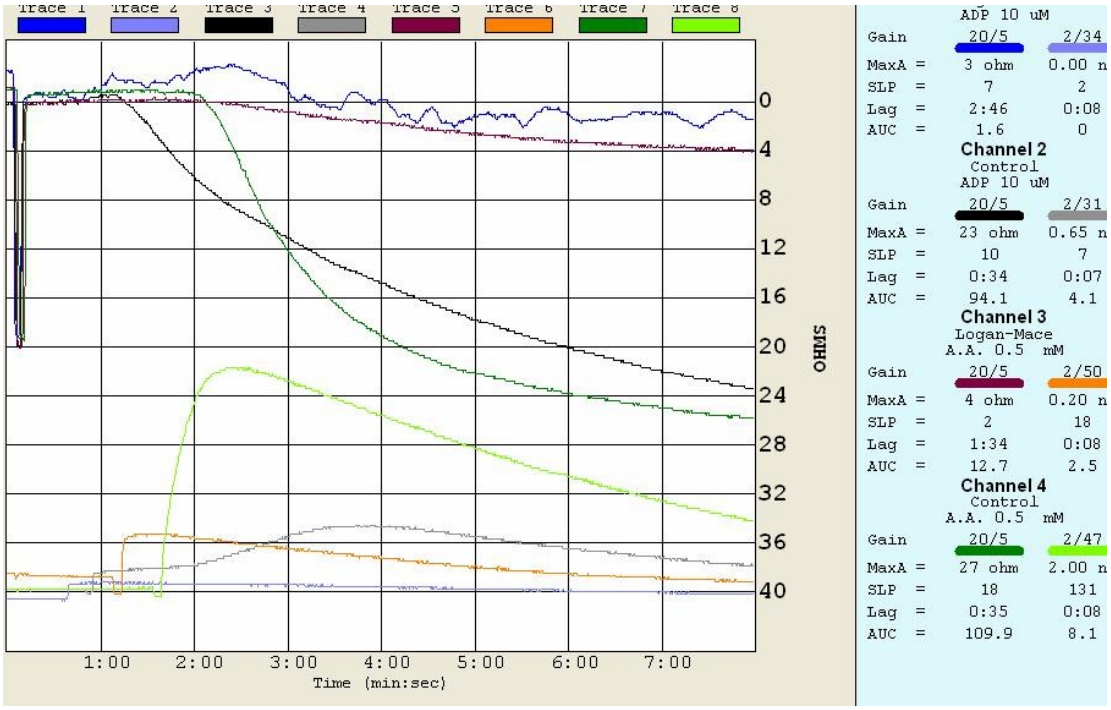


Fig 30: Trace showing Chronolog whole blood aggregation (top) and release reaction (bottom) to collagen, ADP and Arachidonate.

Whole blood aggregometry is normally performed in conjunction with luminescence studies. The luminescence component of the assay will provide 'real time' information on the platelets release of ADP. If this is reduced (as in the examples below) then there are two possible pathologies. One diagnosis could be that the enzyme signalling cascade causing the release mechanism is at fault; the other is that the granule contents (or granules themselves) are absent. To distinguish between these platelet nucleotide analysis must be performed (HSP-004 next section) If the nucleotides are present then the former diagnosis is the most likely, if not the second is.





Figs 31 & 32: Above are examples of suspected storage pool disorder patients analysed with lumi-aggregometry. Note the reduction in the release curves.

In Figs 31 and 32 there is a marked reduction in the release curves when compared to those in Fig 30. In Fig 32 the green lines show the aggregation (dark green) and release (light green) of the normal control to arachidonic acid. In comparison the patients' aggregation (purple) and release (orange) are reduced indicating reduced release. This could be because the granule products are not being released (enzyme defect) or there is none to release (dense granule defect).

Platelet nucleotides will distinguish between these.

2.2 Platelet nucleotides. (HSP-004)

Depending on the aggregation results, as mentioned above, will inform the direction of further testing. One of the most common primary specific platelet disorders is delta storage pool disorder. This will be characterised by a biphasic response to ADP at high concentrations and first phase with reversal at lower concentrations in LTA and weak release and response in WBA. To separate delta storage pool disorder from the rarer enzyme disorder platelet nucleotide analysis is done. This measures the ATP and ADP in the delta (dense) storage granules in the platelet. There are 5-6 dense granules per platelet and they also contain:

GPIb		GPIIb-IIIa	LAMP2
CD-63	5-HT		Calcium Ions
CD-62P	Histamine		GTP/GDP
Magnesium Ions			

If the platelet nucleotides levels and the ratio is outside the reference range this is indicative of a storage pool disorder.

If the nucleotides are normal then this could indicate a release disorder. If platelet nucleotides are being released re-assay the patient. However if the patient does not release the nucleotides in the whole blood assay this indicates that the mechanism for releasing the granules into the environment is at fault.

2.3 Platelet Lysates (HSP-009)

Platelets also have larger more common granules (approximately 40/platelet) known as alpha granules. These are the granules which stain giving platelets on a blood film their granular appearance. Hence when they are absent the disease is referred to as ‘grey platelet syndrome’. Laboratory diagnosis of this disorder is based around analysis the contents of these granules, and electron microscopy. The easiest of the alpha granule content to analyse is fibrinogen and VWF (other contents are shown in the list below)

P-Selectin (CD-62P)	GP IIb-IIIa	GPIb-IX	
Fibronectin	PECAM	beta-Thromboglobulin	
Vitronectin	PF-4	GPIV	
Thrombospondin	VWF	PDGF	EDGF
Fibrinogen	Kininogen	Protein S	VEGF
Factors V, VIII, XI, & XIII		Plasminogen	

Platelet glycoproteins (HSP-005)

Analysis of the surface receptors is diagnostic in diseases such a GT and BSS.

GT	Type I	0-5% functioning GPIIb/IIIa
	Type II	6-20% functioning GPIIb/IIIa
	Variant	21-100% with variable fibrinogen binding

BSS

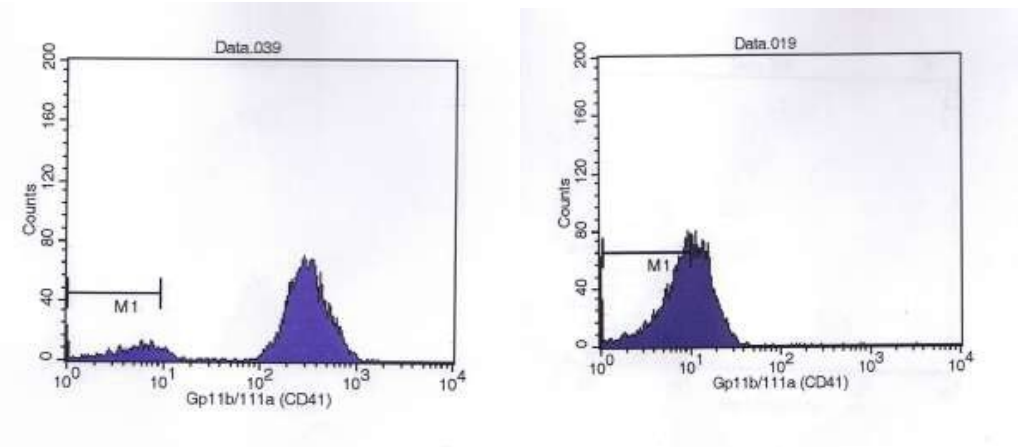


Fig 33; CD41 (GPIIb/IIIa) FACS results, normal control histogram on the left and a Glanzmann's patient on the right

As this test is specific, quantitative test there is no information that can be 'infered' from the results. The patient either has a deficiency or they have normal levels of glycoprotein.

Ristocetin induced platelet aggregation

Type IIb von Willebrand's disease is characterised by the absence of large VWF multimers from plasma, and induction of platelet aggregation in platelet rich plasma (PRP) at concentrations of ristocetin lower than required for normal individuals. The high molecular weight (HMW) multimers are absent from the plasma due to a mutation that causes the VWF to have an increased affinity with GPIb-IX receptor. This can lead to thrombocytopenia due to platelet aggregates being removed from the circulation.

Thus, this technique, in combination with VWF multimer analysis, supports the diagnosis of Type IIb von Willebrand's disease. Additionally, pseudo-VWD (or platelet type VWD), that has a similar pathology, is hyper-responsive to ristocetin due to a mutation causing an increased sensitivity of the patients GPIb to normal VWF.

These diseases give a 'gain of function' in that the platelets over react to ristocetin. Therefore the assay uses dilutions of ristocetin to establish that this is the case. Once this is confirmed the use of 'mixing studies' is used to determine which of these diseases it is. Mixing studies is the process of washing the patient and control platelets and resuspending them in their opposite plasma. Patients' platelets in control plasma and control platelets in patients' plasma, then the RIPA is performed again. If the over reaction is in the sample with patients platelets it is Pseudo (platelet type) VWD and if its patients plasma then is VWD Type 2b.

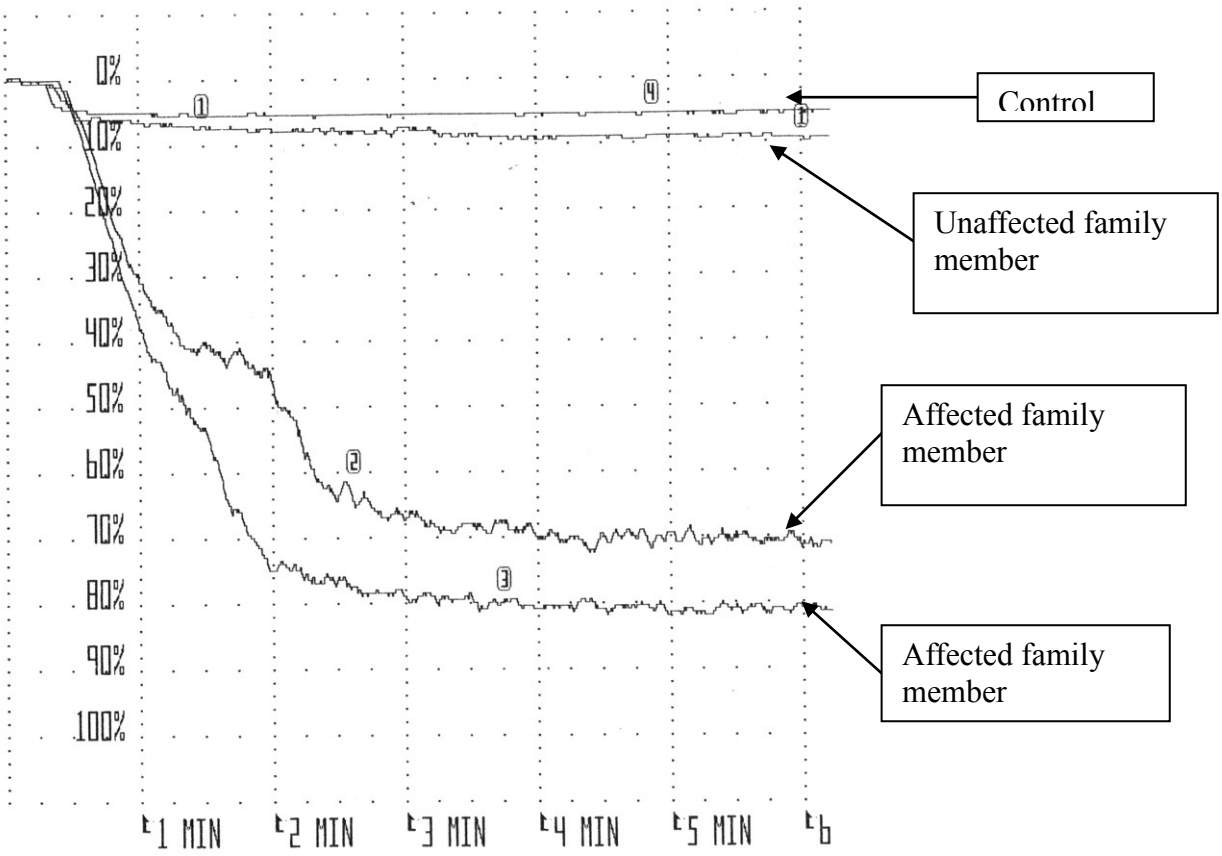


Fig 34: Platelet aggregation with 0.5g/L Ristocetin (normal screening concentration 1.25g/L) showing control, unaffected family member and two affected family members

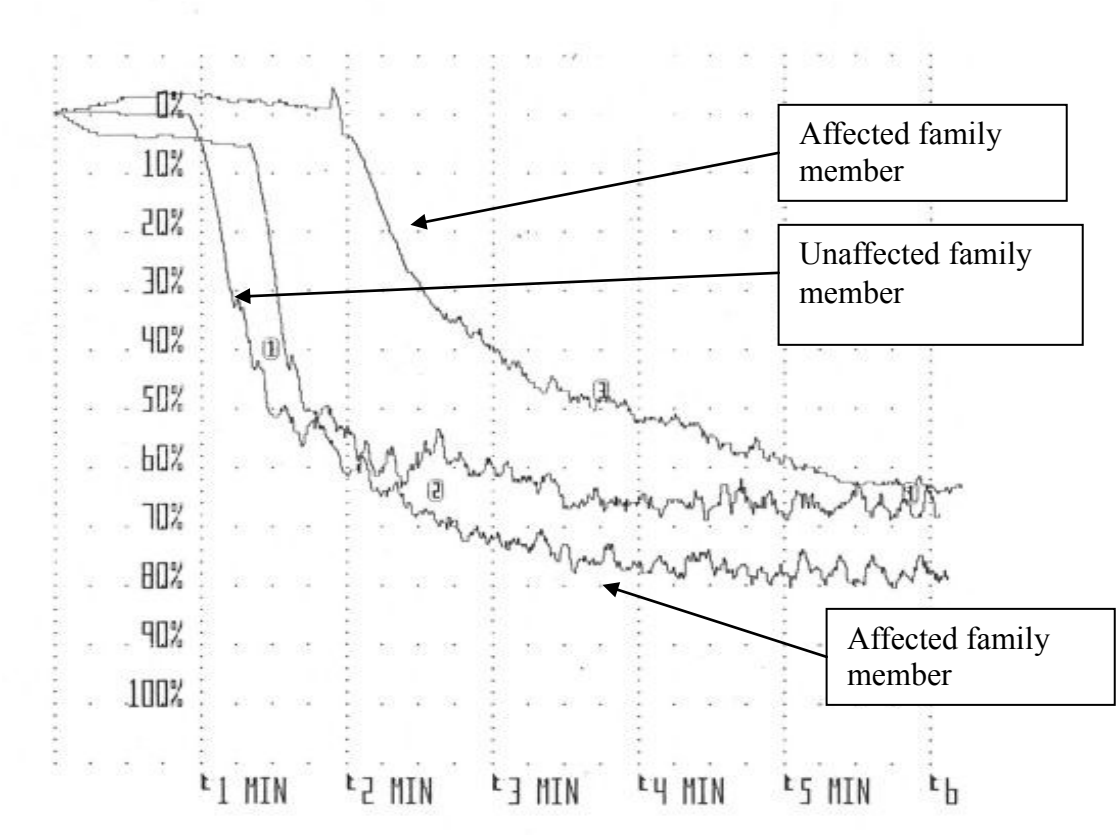


Fig 35: Mixing studies showing above family platelets suspended in control plasma stimulated with 1.25g/L Ristocetin (normal) Notice all patients respond.

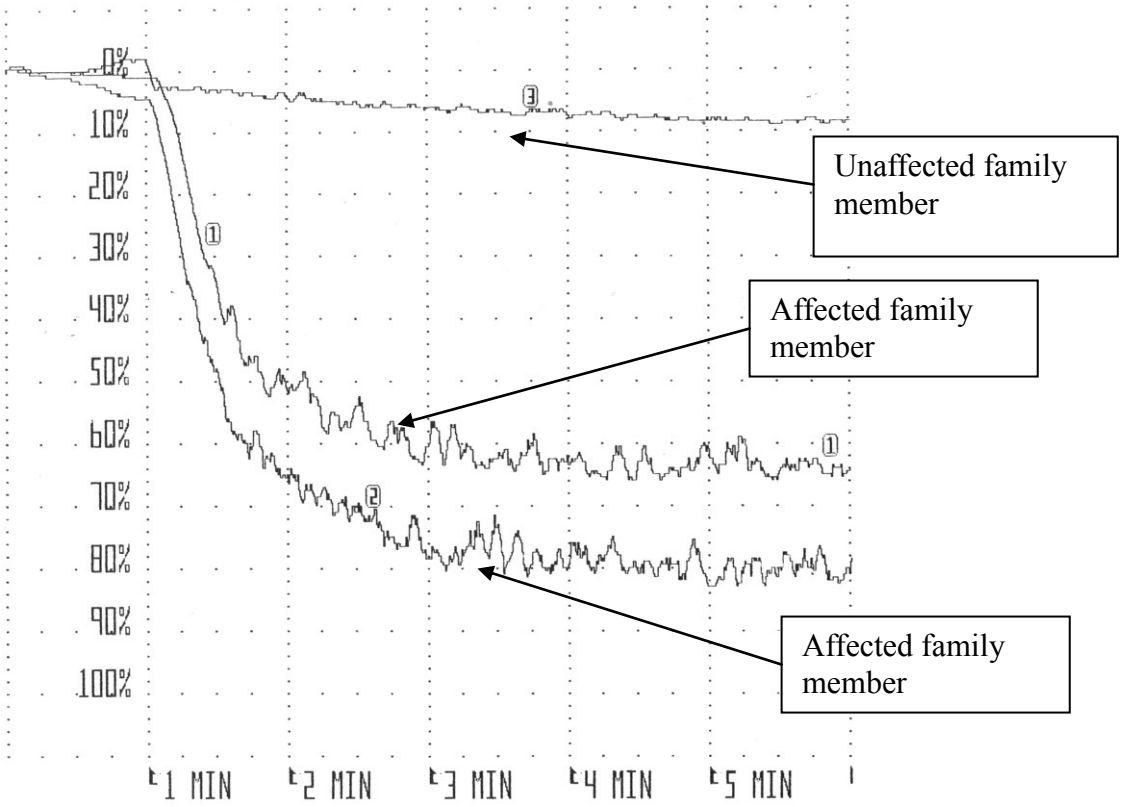
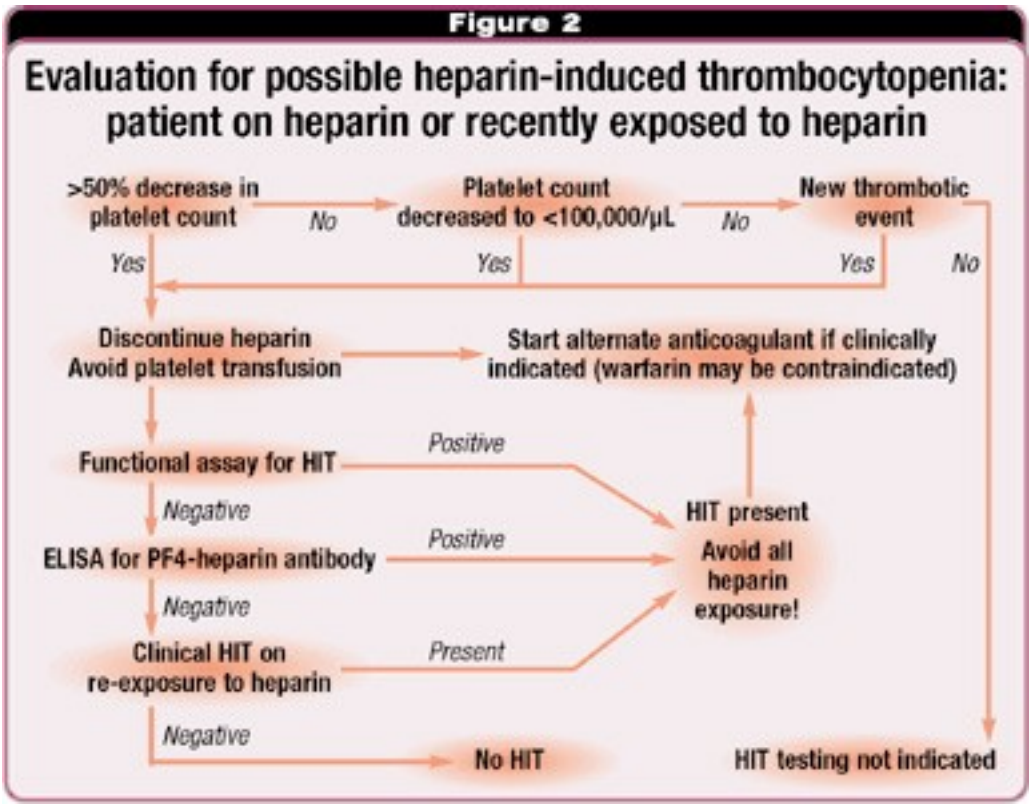


Fig 36: Mixing studies showing above family platelets suspended in control plasma stimulated with 0.5g/L Ristocetin (low dose)

Heparin Induced Thrombocytopenia (HIT)



Guidance for HIT diagnosis
From http://www.cap.org/apps/docs/cap_today/images/maycoag2.jpg

Patients receiving heparin treatment for at least a week may develop thrombocytopenia. In some cases the platelet levels reduce moderately and return to normal even when heparin treatment is continued. This type of thrombocytopenia is termed Type I heparin-induced thrombocytopenia (HIT) and is not antibody mediated. In other patients thrombocytopenia is more severe and is antibody mediated. This condition is termed Type II HIT. It is known that antibodies associated with Type II HIT recognise sites on a platelet protein 'platelet factor 4' (PF4) that are created when PF4 is complexes with heparin. Two types of assay are available to diagnose type II HIT: functional and antigenic assays. No one type of assay is 100% reliable for the diagnosis of HIT**.**

Serotonin Release Assay (SRA) Not done at GSTS.

The serotonin release assay is the 'gold standard' for diagnosing HIT. In this test, normal platelets obtained from a donor previously shown to be reactive to a panel of heparin-PF4 antibodies, are incubated with radio-labelled serotonin. The labelled platelets are then incubated with the patient's serum in the absence and presence of heparin (in therapeutic and supratherapeutic concentrations). Platelet activation is measured by the release of radioisotope from the platelets.

Heparin Induced Platelet Aggregation (HIPA) (HSP-007)

Platelet aggregation for HIT testing follows similar principles as the SRA. Donor platelets are exposed to patients' plasma in the presence of two different concentrations of heparin. In this case the end point is measured via platelet

aggregation. A positive result is noted when the low dose of heparin (0.5IU) causes aggregation but the high dose (1000IU) does not. The high dose causes an increase in the negative charge present in the solution interfering with the IgG PF4 antibody complex and they cannot react with the platelets Fc receptor.

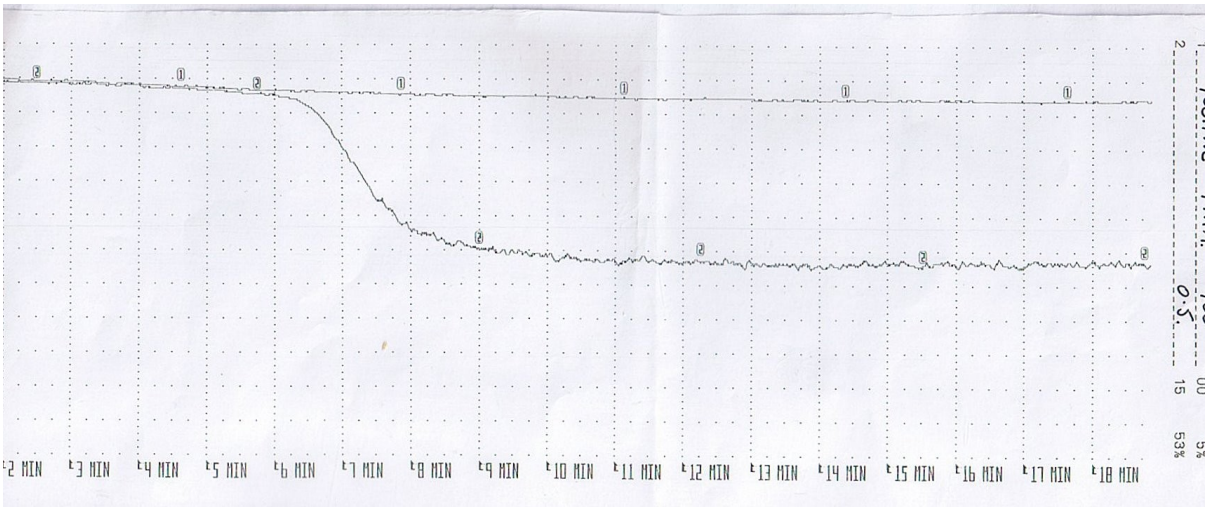


Fig 37: Positive HIT aggregation performed on a PAP-4D

HIT ELISA (HSP-008)

The GTI ELISA is a commercial ELISA kit that measures antibodies to the HeparinPF4 complexes. There are two variations of the kit, one that measures all antibodies (IgG, IgM and IgA) and one that measures IgG alone. The IgG antibodies have been found to be the major causative subtype and most guidelines state that it is only necessary to measure IgG (see *Keeling D, Davidson S, Watson H, Haemostasis and Thrombosis Task Force of the British Committee for Standards in Haematology. The management of heparin-induced thrombocytopenia. British journal of haematology. 2006 May;133(3):259-269* for example)

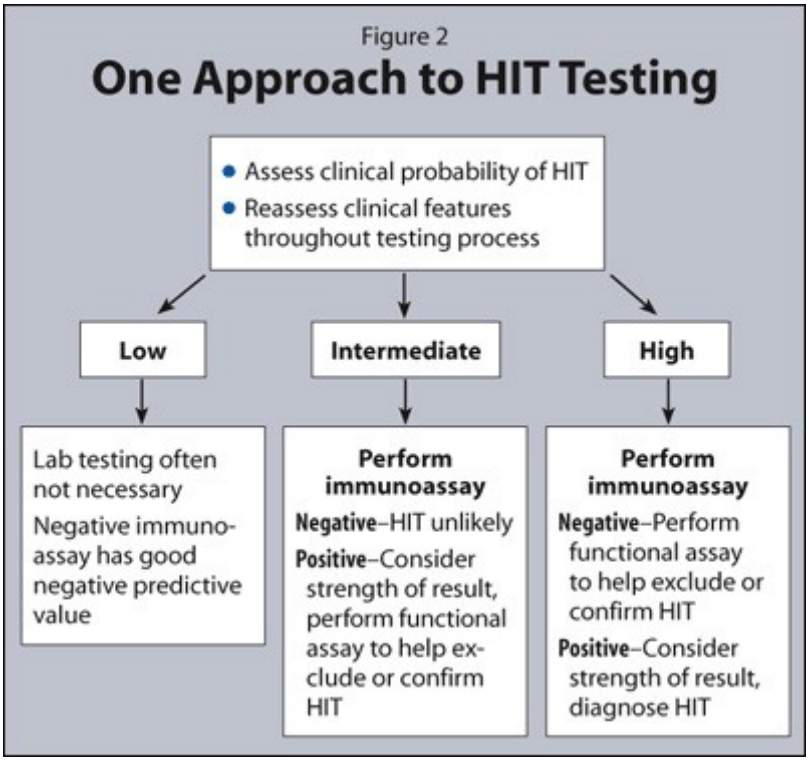


Fig 38: Appropriate use of laboratory HIT assays.
From http://www.aacc.org/publications/cln/2009/May/Pages/series0509.aspx

Interpretation of HIT results.
HIT ELISA assays have a high sensitivity but a poor specificity. That is that they can detect the antibodies, but these antibodies don't always cause HIT. If the patient has a low clinical probability (4T score - *Warkentin TE. Platelet count monitoring and laboratory testing for heparin-induced thrombocytopenia. Archives of pathology & laboratory medicine. 2002 November;126 (11):1415-1423*) and a negative ELISA then the probability of HIT is very low. Alternatively, if the 4T score indicates a high probability and the ELISA optical density (OD) is high (as in on a par with the positive control) then the probability of HIT is high. If neither of these criteria is met then the diagnosis is somewhat more complicated. If the ELISA is not 'clear cut' then a qualitative assay should be performed. Qualitative assays include the SRA and the HIPA. These tests measure the ability of the anti-platelet complexes to activate control platelets.

The paper below gives further guidance
Warkentin TE, Heddle NM. Laboratory diagnosis of immune heparin-induced thrombocytopenia. Current hematology reports. 2003 March;2(2):148-157.

Bleeding time (*not done at GSTS****)***

The bleeding time measures the time taken to stop a specific sized wound to stop bleeding. It has largely been superseded by the PFA-100 and genetic analysis. It has recently become discredited for the variability of the results, but is the only 'rapid' method to measure the part played by the vasculature in the healing process. It has however been discredited in a number of guidelines and papers such as those below:

Peterson P, Hayes TE, Arkin CF, Bovill EG, Fairweather RB, Rock WA, et al. The Preoperative Bleeding Time Test Lacks Clinical Benefit: College of American Pathologists' and American Society of Clinical Pathologists' Position Article. Arch Surg. 1998 February;133(2):134-139.

Lind SE. The bleeding time does not predict surgical bleeding [see comments]. Blood. 1991 June;77(12):2547-2552

Impact-R

The impact R is the commercialisation of a technique first described as the 'cone and plate(let)' method (*Kenet G, Lubetsky A, Shenkman B, Tamarin I, Dardik R, Rechavi G, et al. Cone and platelet analyser (CPA): a new test for the prediction of bleeding among thrombocytopenic patients. British journal of haematology. 1998 May;101(2):255-259*) and is a screening tool is a similar vein to the PFA-100. It has advantages over the PFA in that it takes a lower volume of blood, but needs greater operator intervention.

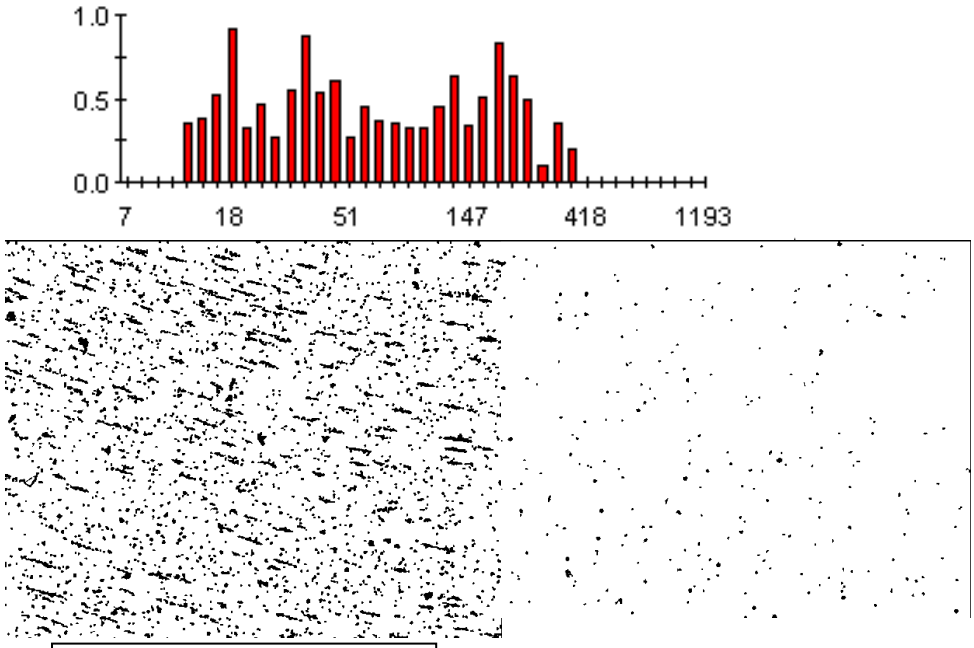


Fig 39: Impact R results from a 'normal' patient

Fig 40: Impact R results for a Glanzmanns Thrombasthenia patient

6.0 Thromboelastogram (TEG)

Thromboelastography or rotational thromboelastographic mapping (ROTEM) work on similar principles and are further attempts to introduce whole blood haemostatic measurements. Both methods rely on the torsion increase in a wire as a clot is formed. TEG rotates a pin attached to the wire and ROTEM rotates the cuvette

with the sample in. The manufacturers of these instruments claim a vast amount of diagnostic information can be gleaned from the traces.

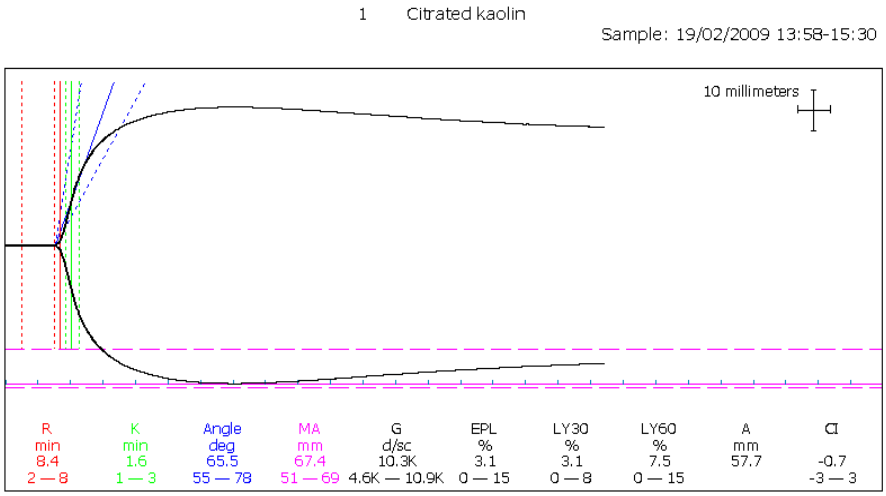


Fig 41: Normal TEG trace

6.1 Genetics

There are many diseases whereby a genetic diagnosis is easier to obtain than a phenotypic one. Among these are the collagen defects such as telangiectasia. More common diseases can be easily subtyped by knowing the specific genetic defect, von Willebrand disease for example.

University of Portsmouth

Application for approval of Professional Doctorate STAGE 2, Part 2:

Professional research and development project arrangements

School of Pharmacy and Biomedical Sciences

Faculty of Science

Academic Year Starting: June 2006

APPLICATION FOR PROFESSIONAL RESEARCH AND DEVELOPMENT PROPOSAL APPROVAL FOR A PROFESSIONAL DOCTORATE IN BIOMEDICAL SCIENCE COVER SHEET

1. Complete the form at all sections, indicating if any are not relevant.

2. The Director of Studies, supervisors, student, Head of Department and Head of Employing Establishment to sign the relevant sections.

3. The Director of Studies to nominate an assessor, from the approved list held in the departmental office, and complete the appropriate section.

4. The completed and signed form to be submitted to Postgraduate Research Officer, Research Office.

5. The Postgraduate Research Officer will ask the Faculty Research Committee (or the Committee representative) to nominate a second assessor.

6. The application is sent to the two assessors, who are asked to make a report and return it to the Research Office.

7. If the reports are in agreement that the student's registration should be approved then a meeting is not required and the Faculty will be notified of the recommendation to approve the full registration. However, if a number of questions arise from the reports, or the assessors wish, a meeting will be arranged to enable the candidate and Director of Studies to discuss the application before a recommendation is made.

8. If any further work or amendments are required following the discussion, these are submitted to the Postgraduate Research Officer who will then liaise with the assessors.

- ☐ Form completed
- ☐ Signatures
- ☐ Ethical approval documentation appended
- ☐ Assessor nominated by Director of Studies
- ☐ Submitted to Research Office
- ☐ Faculty Assessor requested
- ☐ Application sent to assessors
- ☐ Assessors reports received
- ☐ Meeting arranged
- ☐ Recommendation

UNIVERSITY OF PORTSMOUTH

APPLICATION FOR PROFESSIONAL RESEARCH AND DEVELOPMENT PROPOSAL APPROVAL FOR A PROFESSIONAL DOCTORATE IN

Biomedical Science

Please complete in black typescript (not less than 10 point type).
After completion, return the form to the Postgraduate Research Officer, Research Office, University House.

1. THE APPLICANT

1.1 Surname GURNEY

1.2 Forenames David Andrew

1.3 Title (Mr, Mrs, Ms etc) Mr.

1.4 Qualifications

MSc	Biomedical Science
FIBMS	Haematology
CSci	Chartered Scientist

1.5 Full address of place of employment

Haemostasis Laboratories
Centre for Thrombosis and Haemostasis (Haemophilia Reference Centre)
St. Thomas' Hospital
Lambeth Palace Road, London, SE1 7EH

1.6 Telephone, Fax number and e-mail address of place of employment

Telephone	020 7188 2797
Fax	020 7188 2726
E-mail	david.gurney@gstt.nhs.uk

1.7 Home address, telephone number, e-mail address

Elyzium
36 Dunstable Road
Toddington
Beds LU5 6DR

Telephone 01525 630025 Mobile 07747 800638
E-mail davidagurney@yahoo.co.uk

2. PROGRAMME OF PROFESSIONAL RESEARCH AND DEVELOPMENT

2.1 Title

A strategy to extend the scope of platelet function testing will enhance the diagnosis of primary haemostatic disorders and impact patient care.

2.2 Aims of the programme

To improve and enhance detection of primary haemostatic disorders, with particular emphasis on platelet disorders in the clinical haemostasis laboratory by:

Critically reviewing current methodologies for platelet function testing and develop methods of minimising pre-assay variability.

Extension of the most commonly used assay, platelet aggregation, to include additional agonists designed to reduce the frequency of equivocal/borderline result interpretations thereby increasing diagnostic precision.

To evaluate and test a new research analyser, the Diamed Impact-R based on the cone and plate(let) methodology, having the ability to adjust shear rates.

To implement and extend the reference ranges for all the platelet assays based in the laboratory.

To convert the platelet glycoprotein assays from a qualitative measurement to a quantitative measurement and investigate the change in numbers of glycoprotein receptors on the surface membrane in response to common antiplatelet agents such as aspirin.

Considering the impact of enhanced diagnostic strategies on patient care and factors influencing implementation and dissemination of these strategies.

Exploring the implications for professional practice upon adoption of new and modified diagnostic approaches.

2.3 **Proposed plan of work** (This must be contained within three pages and should include: background, stages of the proposed programme of research, outline methodology and key references)

Background

Platelets play an integral role in maintaining normal haemostasis, acting as delivery vehicles and reaction support in addition to their traditional role of 'plugging' the break in the vasculature. More recently the study of platelets and detection of clinically significant abnormalities has developed beyond basic aggregometry utilizing fluorescent conjugated monoclonal antibodies and luminescence reagents. These diagnostic strategies have lead to the requirement of expansion of the present specialist platelet facilities in order to accommodate the equipment and reagents and utilisation of expertise.

Research plan

Phase 1a: Development of existing repertoire.

Time scale: Now - 6months

1. Using thrombin receptor agonist peptide (TRAP), a thromboxane analogue (U-44167) and a calcium ionophore (A23167) as additional agonists the repertoire for platelet aggregation has been expanded significantly. The development and refinement of this procedure will be ongoing.
2. Standardisation of the procedure for producing washed platelets for analysis by Ristocetin Induced Platelet Aggregation (RIPA) has been accomplished. This was implemented as the technique was used rarely and needed updating. It is crucial for detecting and distinguishing between VWD Type IIb and Pseudo VWD, an essential part of a reference centres repertoire
3. The luminometer that is used to analyse platelet nucleotides is being replaced, the analysers from Thermo Life Sciences and Berthold Industries having been evaluated, the Berthold analyser has been purchased and now needs assimilation into laboratory procedure including writing of standard operating procedures, generating reference ranges and staff training. This will lead to a direct improvement in assay quality and turn around time.
4. The development and standardisation of the platelet glycoprotein method using FACScan analysis is on-going. Microspheres of a known size coated with relevant antibodies to produce a standard curve of fluorescence. This graph is then used to quantify fluorescence on

the platelet membrane surface.
5. The implementation of a pre-analytical questionnaire for platelet aggregation has reduced the reporting of false positives and minimised inappropriate testing due to the patient taking medications known to affect platelet aggregation

Phase 1b: Development of new reference ranges for existing repertoire
Time Scale Concurrent with Phase 1a
The development of platelet aggregation reference ranges for all the new agonists in the aggregation repertoire and reaffirmation of the ranges for the established agonists. Nucleotide reference ranges are to be re-established at the same time

Phase 2: Development of Glycoprotein Reference Ranges.
Time Scale: Concurrent with Phase 3 (Three years)
Development of antibodies to a range of glycoproteins and flow cytometric counting technology has led to being able to count the number of glycoprotein sites that have bound the fluorescent antibodies. Whereas this has aided diagnosis and discovery of abnormal states, there has not been an establishment of a reference range. Addressing the development of a reference range for the standard platelet glycoproteins would be a priority of the new laboratory. This would entail development of the counting technology on the current BD FACScan analysers, involving the calibration of Calibrite beads, and establishing the antibodies to be used for further investigation. Assessment of the Quantum Simply Cellular Bead system and implementation for routine glycoprotein assays is also planned.
The minimum number of samples for establishing a range is normally accepted as twenty. For this to be able to stand as a standard then at least double that number need to be recruited and ideally one hundred normal volunteers recruited. These volunteers need to be strictly monitored for dietary/pharmaceutical agonists such as aspirin, anti-depressants, green tea, smoking and vitamin B6. These patients will have a glycoprotein panel run and data analysis completed.

Phase 3a: Monitoring patients against the new reference ranges.
Time Scale: Concurrent with Phase 3b (Three years)
Every patient that comes in for platelet function analysis will have flow cytometric analysis of the glycoprotein receptor numbers. Past patients that have had ambiguous platelet results would be reinvestigated within the context of the new normal ranges to determine whether these patients have significant variation from the numbers of platelet glycoprotein sites available and whether these results contribute to their pathology.

Phase 3b: Monitoring effects of external agents on normal range
Time Scale: Concurrent with Phase 3a
Investigation into whether drugs and dietary supplements, such as those mentioned above, which are known to affect platelet function, also affect glycoprotein receptor numbers. Volunteers (that are known to be taking certain drugs/dietary supplements) would be monitored over a period of three months to determine basal levels and to establish whether the products reduce the receptor numbers.

Stage 4. Professional dimensions

4.1 Dissemination of research findings

If the research findings are to have more than a local impact, they must be disseminated in such a way as to reach as wider audience as possible. Implementation will include seminars within the department, revision of SOPs and publication in trade and peer reviewed journals.

4.2 Local and wider implementation new methodologies

All reference ranges, new technologies and new assays will be implemented within the department. The objective is to make the department a centre for excellence in platelet

diagnostics. To enhance this reputation methodologies and modifications will be published in trade and peer reviewed journals.

4.3 Impact on patient care of adoption of new methodologies

40% of all primary haemostatic disorders remain without a sufficient diagnosis. The aim of the centre of excellence is to increase this diagnostic 'hit rate'

4.4 Personal reflection

Reflection will be undertaken covering the personal development achieved as a result of undertaking the research and having contributed to departmental research output from a diagnostic laboratory.

References

2.4 Facilities in the Department/Faculty/University which will be used in support of the programme

All research will be accommodated in the workplace. University staff will be closely involved in guidance (i.e. Dr David McLellan, Dr Graham Mills).

2.5 Facilities in the Workplace which will be used in support of the programme

(i) Establishment/Individual(s):

Key members of staff:	Dr S Rangarajan	Consultant Haematologist
	Dr GW Moore	Head Biomedical Scientist
Other staff:	Biomedical Scientist colleagues in the Haemostasis Laboratories	

(ii) Facilities available:

Use of laboratory facilities, bench space and basic equipment.

Reagents and consumables

Analytical equipment:
PFA-100 platelet function analyser
PAP-4 Platelet aggregometer
Chronolog Whole blood aggregometer
BD FACScan
Berthold Lumat LB9507 Luminometer
Diamed Impact R

IT hardware and software

2.6 Details of collaboration required for programme

None

2.7 Has ethical approval been applied for or obtained at your place of work/employer?
Note: *All research subsequently undertaken for this unit must conform to the principles embodied within the Declaration of Helsinki (October 2000)* (Give full details of the application, reference number and dates. If ethical approval will not be sought, please justify why this should not be the case). Ethical Committee decisions **must** be obtained before the commencement of any research work; and copies of these decisions where already available should be appended to this form. In addition, your proposal may be presented to the University of Portsmouth's Ethical Committee for further approval.

Ethical approval has been given for platelet glycoprotein analysis for a previous project. This project is building on that data and the same approval holds.

Ethical approval has **not been sought** for the following reasons:

1. The samples that are taken for routine platelet analysis are consented as a matter of course. Part of the project involves assessment of additional platelet diagnostic assays, thus implied consent has been given[1]

2 As no additional blood samples are taken and results generated are within the remit of platelet diagnostics this represents no additional risk to the patient.[2]

3. The department has a consent process in place for donation of blood samples for reference ranges and normal controls.

References

1. Transitional guidelines to facilitate changes in procedures for handling ‘surplus’ and archival material from human biological samples. Royal College of Pathologists, London. 2001.

2. Human tissue and biological samples for use in research: operational and ethical guidelines. Medical Research Council, London. 2001.

3. COST OF THE PROGRAMME OF WORK

FINANCIAL DETAILS OF SUPPORT REQUIRED:

1. Associated Staff	Year 1	Year 2	TOTAL
Grade % FT Salary Employer Contribution	**Some results from initial diagnostic investigations have been generated by incumbent Biomedical Scientists.** **No additional costs**	**Some results from initial diagnostic investigations have been generated by incumbent Biomedical Scientists.** **No additional costs**	
Subtotal			
2. Materials and Consumables			
Platelet aggregation agonists **PFA Cartridges** **Impact R Reagents** **Glycoprotein antibodies & kit** **Buffers for FACS analyser** **Laboratory consumables – pipette tips, cuvettes etc**	**£1000** **£100** **£200** **£600** **£25** **£100** **£300**	**£1000** **£100** **£200** **£600** **£25** **£200**	
Subtotal	£2025	£2125	£4250
3. Equipment			
PFA-100 PAP-4 PRP aggregometer Chronolog Whole blood aggregometer BD FACScan Berthold Lumat LB9507 Luminometer Diamed Impact R **IT hardware & software**	**All equipment part of establishment – no additional costs**	**All equipment part of establishment – no additional costs**	
Subtotal			
4. Travel			
Visits to the university	**£100**	**£150**	

Subtotal	£100	£150	£250
5. Other e.g. animals			
N/A			
Subtotal			

Total costs £4500

Reasons for support required:

Please justify a) staff, b) material and consumables, c) equipment and d) animals required. (Continue on an additional page)

Staff

The Biomedical Scientist staff involved have generated any results used as part of their normal duties. These results constitute those necessary for initial diagnosis to confirm the presence of disease states relevant to the project. All other investigations, modifications, development and assessment have, or will, be performed by the applicant.

Materials & consumables

PFA/Aggregation

The PFA-100 uses a cartridge based system to process samples. This analyser is in routine use in the department and consequently consumable cost will be absorbed into the normal department routine. The reagent panel for platelet aggregation is being extended and as a consequence additional reagents are being ordered. This increases the consumables being used with the test. Once this is a proven technique it will be incorporated into the departments' repertoire and funded accordingly.

Flow Cytometry

Flow cytometry is in routine use within the department, this is just an extension of this method. Additional antibodies will be purchased for the project using different antigenic targets on the platelet surface using known fluorochromes. Using supplementary reagents these fluorescence levels can be calculated to express numbers of surface glycoproteins. A company has provided the project with a discount code for ordering these antibodies and reagents. Once this is a proven technique it will be incorporated into the departments' repertoire and funded accordingly.

Diamed Impact-R

The Diamed Impact-R is a relatively new analyser that the department is evaluating with a view to purchasing to widen its diagnostic range. This evaluation procedure would have been undertaken by the department for any new procurement of equipment. It is therefore of no additional cost both in terms of instrument (as it is on loan for the trial) or reagents, as they would have been evaluated as a matter of course.

Luminometry

The departments' luminometer had come to the end of its working life and it was established that the results it produced were integral to our diagnostic service. The department is evaluating with a view to purchasing this replacement. This evaluation procedure would have been undertaken by the department for any new procurement of equipment. It is therefore of no additional cost both in terms of instrument (as it is on loan for the trial) or reagents, as they would have been evaluated as a matter of course.

Travelling

It will be necessary to visit the university for meetings and tutorials with supervisors. Travelling to conferences and meetings may be necessary as part of the dissemination process.

Please give any details/dates of external funding **obtained to conduct this investigation. Has external funding been sought?**

No external funding has, or will, be sought.

How will the financial costs be met? (Give details of how the project will be funded)

The project will be funded entirely by the department; however we have managed to secure discounts on reagents from some manufacturers

3.1 Associated Staff

Within current staffing budget.

3.2 Consumables and Materials

Within reagent budget for the Diagnostic Haemostasis Laboratories.

3.3 Equipment

All necessary equipment is available within the department.

3.4 Travel

Within departmental expenses budget.

3.5 Other e.g. animals

N/A

4. EXPLOITATION AND DISSEMINATION OF RESULTS

1. If the proposed research is likely to give rise to a commercially viable discovery please give details, including an indication of the likely route for transferring technology to the market place.

   This is a proposed evaluation of change and extension of practice. New technology/ developments are unlikely to arise.

2. If no immediate commercial benefit is foreseen, applicants should give details of how the knowledge gained from the project will be passed to the public domain.

   It is intended that all the work will be presented for publication in appropriate medical / biomedical science journals. The work will also be summarised and presented for publication in the professional gazette of the IBMS and may be included as required reading for specified CPD activities. Presentations at scientific meetings are planned and also an approach to a scientific standardisation committee for inclusion in future guidelines.

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5. STATEMENT BY APPLICANT

I wish to apply for registration for a programme of professional research/development leading to a Professional Doctorate; the proposed start date is 01/07/00

I confirm that the particulars given at Section 1 are correct. I understand that all research work undertaken must confirm the ethical principles embodied within the Declaration of Helsinki 2000. I understand that, except with the specific permission of the University, I must prepare and defend my portfolio/thesis in English.

Signature of Applicant ..... Date .....

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6. RECOMMENDATION BY SUPERVISORS

We support this application and believe that Mr. David Andrew Gurney has the potential to complete successfully the programme of work proposed. We recommend that this applicant be registered for the University's Professional Doctorate degree.

Signature Date:

Signature Date:

Signature Date:

Signature Date:

Signature Date:

Comments:

7. SUPERVISORS

7.1 Director of Studies

Name: Dr David McLellan

Qualifications: PhD MSc FIBMS MIBiol

Current post/where held: Honorary Lecturer, University of Portsmouth

I am currently supervising: 2 student(s) as Director of Studies, and no student(s) as an Additional Supervisor.

Previously supervised & completed: MPhil - 2 PhD - 2 PD - 2

7.2 University Supervisors (with area of expertise/responsibility)

Name: Dr Graham Mills

Area of Expertise Professional Doctorates

Area of Responsibility Course Manager for DBMS programme

Qualifications:

BSc(Eng) BA(Ed) CertEd MA(Ed) PhD CChem FRSC CEng MinstE AMI Chem E

Current post/where held Principal Lecturer, University of Portsmouth
Telephone No: 02392 842115

Current Supervision: 2 x PhD, 1x PD

Previously supervised/completed:

MPhil - 0 PhD - 3 PD - 1

7.3 Workplace Supervisors/Advisors (with area of expertise/responsibility)

Name: Dr Gary Moore

Area of Expertise Haemostasis

Area of Responsibility Supervisor

Qualifications: BSc DBMS CSci FIBMS CBiol MIBiol CertMHS

Current post/where held
Principle Biomedical Scientist
Haemophilia Reference Centre
Centre for Haemostasis & Thrombosis
St. Thomas' Hospital, London, SE1 7EH

Telephone No: 020 7188 2797

Current Supervision:

Previously supervised/completed:

MPhil - 1 MSc - 4 PD - 0

Name: Dr Savita Rangarajan

Area of Expertise Haematology

Area of Responsibility Advisor

Qualifications: MRCP MRCPath

Current post/where held
Consultant Haematologist
Haemophilia Reference Centre
Centre for Haemostasis & Thrombosis
St Thomas' Hospital, London SE1 7EH

Telephone No: 020 7188 2806

Current Supervision:

Previously supervised/completed:

MPhil - 0 PhD - 0 PD – 0

7.4 Advisors

Name:

Qualifications:

Current Post:

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8. HEAD OF SCHOOL/DEPARTMENT

I have accepted this student in the department and confirm that the facilities, details given in Section 2.3, will be provided.

Name:…….

Signed:..... Date .....

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9. HEAD OF EMPLOYING ESTABLISHMENT

I confirm that the facilities, details given in Section 2.5, will be provided. I confirm that ethical clearance has been sought/approved to undertake the proposed work within the establishment.

Name: Dr Savita Rangarajan

Signed:.....…..... Date .....

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10. ASSESSOR(S) NOMINATED BY DIRECTOR OF STUDIES

Assessor:

Department:

Telephone No:

Assessor:

Department:

Telephone No:

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11. ASSESSOR NOMINATED BY THE FACULTY RESEARCH COMMITTEE

Assessor:

Department:

Telephone No:

12. APPROVAL OF REGISTRATION

I confirm that the candidate was registered for the degree of Professional Doctorate with effect from:

Signature: ..... Date:
(Chair/Secretary of Research Degrees Committee)